THE ROLE OF STRESS IN RECOVERY OF FUNCTION AFTER SPINAL CORD INJURY

A Dissertation

by

STEPHANIE NICOLE WASHBURN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Psychology

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Approved by:

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ABSTRACT

The Role of Stress in Recovery of Function after Spinal Cord Injury. (August 2007)

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Chair of Advisory Committee: Dr. James W. Grau

Research has shown that exposure to just 6 minutes of uncontrollable shock 24 hours following contusion injury impairs locomotor recovery and leads to greater tissue loss at the injury epicenter. Uncontrollable shock is known to elevate corticosterone levels in intact rats and corticosterone exacerbates cell death in the hippocampus following injury, suggesting the effects may be related to a stress-induced release of corticosterone. Uncontrollable shock also affects other indices of stress including, spleen weight and norepinephrine, and has been shown to elevate pro-inflammatory cytokines. The present experiments were designed to assess whether uncontrollable shock has similar effects after contusion injury.

Experiment 1 examined whether injury itself produced a stress response. Subjects received anesthesia alone, a laminectomy, or a contusion injury. Twenty-four hours later, they were restrained for 6 minutes and blood was collected from the leg. They were sacrificed 24 hours later and spleens were weighed, and plasma corticosterone and norepinephrine were assessed using ELISAs. IL-1β and IL-6 levels at the injury site were also measured using an ELISA. Contusion injury had no impact on any of the biological outcomes. For Experiment 2, subjects received 6 minutes of uncontrollable tailshock or an equivalent amount of restraint. Subjects were sacrificed 6,

24, 72, or 168 hours later. Uncontrollable shock caused a decrease in spleen weight and increased plasma corticosterone within 24 hours. Increases in IL-1β and IL-6 were also seen. Morphine was used in Experiment 3 to block the "psychological" component of uncontrollable shock. Subjects received morphine (20 mg/kg; i.p.) or saline 30 minutes prior to uncontrollable shock and were sacrificed 24 hours later. Morphine did not prevent the consequences of uncontrollable shock and, in some cases, potentiated its effects. The effect of controllability was examined in Experiment 4. After receiving a contusion injury, subjects received either controllable (master) or uncontrollable (yoked) legshock over the course of 2 days. A third group served as unshocked controls. Master subjects did not differ from yoked subjects on any of the biological outcomes measured. Unshocked subjects, however, exhibited an increase in corticosterone, IL-6, and blood monocytes.

DEDICATION

I am blessed to have met so many wonderful people over the course of my graduate career. This dissertation is dedicated to all those people who supported me through it all. Among them are Chris Williams and Denise Puga who were always there to help me drown my sorrows and celebrate my successes. They never let me give up. I am truly grateful for their friendship and hope we remain friends for years to come. Roland Romero has given me invaluable encouragement and advice over the past 5 years. I truly couldn't have done this without him. Adam Ferguson has been my savior since the day I arrived in the Grau lab. He has served as my mentor and surrogate big brother from the beginning. There are no words to express my gratitude. James Courtney gave me a new perspective on life that kept me from going crazy throughout the dissertation process. He reminded me of the importance of family and I am so excited about our new family. I love him and my new "sons" very much. Last but certainly not least, I dedicate this dissertation to mom and dad who have been my rock. Growing up they always told me that I could accomplish anything I put my mind to. Even when I wasn't sure, they never gave up on me and always believed this day would come. I can't express how thankful I am to have such great parents. I am who I am today because of them. I love you very much mom and dad!

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CHAPTER I

INTRODUCTION

Spinal cord injury is a devastating event characterized by paralysis below the site of injury, loss of bladder and bowel function, and in many cases, the development of neuropathic pain. According to the Christopher and Dana Reeve foundation website, there are approximately 250,000 people currently living with a spinal cord injury, with about 11,000 cases reported each year. The majority (> 50%) of these people are between the ages of 15 and 29 years old. The fact that spinal cord injury occurs early in life makes it especially difficult for the individual financially, as well as, emotionally. It is estimated that the lifetime cost for an individual injured at the age of 25 is $2.8 million and overall, cost the United States an estimated $9.7 billion each year (Berkowitz, 1998). However, these figures only account for the direct medical costs associated with the injury and does not include costs for mental health care. Approximately 30% of patients will have at least one major depressive episode as a result of the loss of function and chronic pain associated with spinal cord injury (Elliot & Frank, 1996). On average, these individuals exhibit less function than non-depressed patients. The reason for this may be motivational, however recent research from our laboratory suggests that both the psychological and physiological aspects of injury may contribute to loss of function.

This dissertation follows the style and format of *Behavioral Neuroscience*.

Using an animal model, we have shown that a commonly used stressor, uncontrollable stimulation, undermines recovery of function after spinal cord injury (Grau et al., 2004). This loss of function appears to result from tissue loss at the injury site, however the mechanism through which this occurs remains unknown. My dissertation was designed to further assess the impact of stress on recovery of function after injury. Specifically, it examines whether uncontrollable shock engages physiological systems related to the stress response and thereby potentiates secondary damage after spinal cord injury.

To understand how uncontrollable shock may potentiate secondary damage it is essential to understand the injury process. In the subsequent sections, I will outline the injury process, beginning with the acute phase and continuing into the chronic stages. Next, I will discuss how stress can affect these processes.

Injury Process

Spinal cord injury follows a stereotypical progression of biological changes that consist of vascular, biochemical, inflammatory, cellular and molecular events. These changes begin at the moment of impact and persist for months. To better understand the injury process, researchers have broken it down into 3 stages consisting of the acute stage, secondary or subacute stage, and chronic stage. The acute stage defines the first 24 hours following the injury. It is a critical stage because what happens during this timeframe can dramatically alter the processes involved in the secondary and chronic stages. The first notable event during the acute stage is the development of gray matter hemorrhage. This area of hemorrhage continues to spread both rostrally and caudally

and ultimately extends into the white matter within hours after injury (Dumont et al., 2001; Norenberg, 2004; Osterholm, 1974). This expanding area of hemorrhage is accompanied by edema and subsequent ischemia. Vasogenic edema causes a break down of the blood-spinal cord barrier and is believed to result in diminished blood flow to the injury site (Griffiths & Miller, 1974). Ischemia, which often results from edema, is thought to initiate a cascade of secondary events that culminate in excitoxicity (Amar & Levy, 1999; Carlson & Gorden, 2002; Choi & Rothman, 1990; Rothman & Olney, 1986; Tymianski & Tator, 1996). The term excitotoxicity was developed to describe the neuronal injury and cell loss that result from excessive glutamate receptor activation (Dumont et al., 2001). The first step in excitoxicity is ATP-depletion and the subsequent dysfunction of energy-dependent processes that maintain cellular homeostasis. This loss of homeostasis leads to an ionic dysregulation that causes swelling of the cell and changes in membrane polarization that promote the release of excitatory amino acids, such as glutamate, from synaptic vesicles (Amar and Levy, 1999; Hulsebosch, 2002). Furthermore, inactivation of cellular uptake mechanisms in both neurons and glia due to hypoxic ATP-depletion contributes to the accumulation of extracellular glutamate (Amar & Levy, 1999). This accumulation of excitatory amino acids in the extracellular space can reach toxic levels within 15 minutes after injury (Hulsebosch, 2002). A number of other neuroactive substances, which are also believed to contribute to secondary damage, are also released acutely in the spinal cord in response to injury including, norepinephrine (Osterholm & Matthews, 1972), histamine (Naftchi et al., 1974),

dopamine (Faden et al., 1981), serotonin (Brodner & Dohrmann, 1977; Liu et al., 1990) and dynorphin (Faden et al., 1985).

Binding of glutamate to NMDA receptors allows the influx of calcium into the cell, which initiates a number of calcium-dependent processes, such as the production of free radicals and lipid peroxidation (Amar & Levy, 1999). If levels of intracellular calcium remain elevated it results in what has been deemed the "final common pathway of toxic cell death in the CNS" (Dumont et al., 2001) and is considered "one of the most significant pathophysiological events after spinal cord injury" (Yezierski, 2002). This destructive process is initiated within 8 hours after injury (Beattie et al., 2002) when high intracellular calcium concentrations activate the intrinsic pathway of apoptosis. Activation of this pathway causes mitochondrial damage and release of cytochrome c. Cytochrome c couples with apoptosis activating factor-1 (Apaf-1) to activate the inducer caspase, caspase-9, which subsequently engages the effector caspases, caspase-3 and –6 to induce cell death (Dumont et al., 2001; Okonkwo & Stone, 2003).

During apoptosis, cells exhibit condensation of chromatin in the nucleus, nuclear shrinkage, and DNA fragmentation with intact membranes and organelle structure. An apoptotic cell eventually shrivels, pulls away from the surrounding cells, and is cleared through phagocytosis. Unlike necrosis, apoptosis does not produce an inflammatory response (Chu et al., 2002; Lu, Ashwell, & Waite, 2000). Apoptosis has been noted in most CNS cell types, including neurons, oligodendrocytes, microglia, and astrocytes (Dumont et al., 2001; Yong et al., 1998). Apoptosis generally begins within 4 hours after injury and persists at the injury site for 24 hours. Given its delayed timecourse and

the fact apoptotic cell death greatly influences the amount of function retained, apoptosis has been a target for experimental treatments aimed at preserving function.

The damage to axons during the initial physical trauma of spinal cord injury is one of the factors contributing to the loss of oligodendrocytes in the days following injury. The axons are stretched rather than torn which causes damage to the nodes of Ranvier leading to a disruption of axoplasmic flow. This disruption causes axonal swelling and disconnection from the cell body over time (Faden, 1993). The degeneration of axons resulting from the initial insult occurs within the first few hours, followed by the complete breakdown of the myelin sheaths by 1 day postinjury (Blight, 1992; Profyris et al., 2004). This destructive process following the initial mechanical injury results in significant loss of axons, particularly medium and large diameter axons (Rosenberg, Zai, & Wrathall, 2005) and the loss of trophic support to the surrounding oligodendrocytes (Profyris et al., 2004). This contributes to apoptosis in oligodendrocytes, which causes widespread Wallerian degeneration over the course of months following spinal cord injury.

The secondary, or subacute phase, of spinal cord injury begins approximately 48 hours after injury and lasts for about 3 weeks (Days 2-21). During this phase, edema and hemorrhage are still present but begin to subside. Areas of hemorrhage begin to cavitate and the lesion begins to expand, doubling in size within 3 days postinjury, as neurons and glial cells continue to be lost through apoptosis (Liu et al., 1997). By day 7, multiple cavitations are present and cysts begin to develop in the central necrotic region (Carlson & Gorden, 2002; Liu et al., 1997). Over time, the injury site evolves into a

cystic cavity filled with activated microglia and phagocytic macrophages surrounded by reactive astrocytes that form the glial scar (Profyris et al., 2004; Schwab & Bartholdi, 1996). This scar separates the injured tissue from the normal tissue and serves as a barrier to axonal regeneration. At the same time, extensive demyelination occurs so that by day 7, small patches of naked axons are common (Gledhill, Harrison, & McDonald, 1973; Gledhill & McDonald, 1977; Griffiths & McCulloch, 1983; Wakefield & Eidelberg, 1975).

The last stage of spinal cord injury is the chronic phase. In this phase, the injury has stabilized and is undergoing very little additional changes. The central lesion undergoes only modest expansion during this time (Liu et al., 1997). The cavity, which is filled with granular debris, fascicles of small myelinated and unmyelinated axons, myelin fragments, and invading blood vessels (Hausmann, 2003), becomes well defined. The glial scar thickens through the process of reactive gliosis as activated astrocytes surround the cavity, imposing a chemical and physical barrier to regeneration (Bandtlow & Schwab, 2000; Caroni & Schwab, 1988; Chen et al., 2000; Fawcett, 1997; GrandPre et al., 2000; Niederost et al., 1999; Pasterkemp et al., 1999; Wang et al., 2002). The chronic lesion is an ellipsoidal cystic cavity surrounded by activated astrocytes and a peripheral rim of spared axons undergoing demyelination (Beattie et al., 2002; Carlson & Gordon, 2002; Hausmann, 2003). Apoptosis of oligodendrocytes occurs for weeks following injury, contributing to the number of fibers that undergo Wallerian degeneration in the first 6 months (Crowe et al., 1997; Grossman, Rosenberg, & Wrathall, 2001; Li et al., 1999; Schwab & Bartholdi, 1996; Shuman, Bresnahan, &

Beattie, 1997). At 1 month, some of the fibers are completely devoid of myelin but the majority exhibits abnormally thin sheaths that surround normal looking axons (Harrison & McDonald, 1977). Over the course of 3-6 months there is a marked reduction in fiber density indicative of complete degeneration of many fibers (Schwab & Bartholdi, 1996). These fibers retract considerable distances (up to 5 mm) at this time (Hill, Beattie, & Bresnahan, 2001; Oudega et al., 1999; Pallini, Fernandez, & Sbriccoli, 1988).

Immune Response

An immune response is generated immediately following spinal cord injury as endothelial cells within the damaged tissue begin to produce pro-inflammatory cytokines and chemoattractants, which ultimately cause neutrophils to migrate to the injury site (Carlson et al., 1998). Whether this immune response is beneficial or detrimental to recovery remains controversial. For example, both neutrophils and macrophages (peripheral and resident microglia) are thought to contribute to the secondary damage that occurs following spinal cord injury (Bethea, 2000; Popovich et el., 2002; Taoka et al., 1997). However, they can also promote axonal regeneration (Rabchevsky & Streit, 1997; Rapalino et al., 1998). Neutrophil infiltration occurs within 3 hours postinjury (Taoko & Okajima, 2000) and peaks at 24 hours (Carlson et al., 1998). Once there, neutrophils produce cytokines that stimulate leukocyte chemotaxis and activate glia (Profyris et al., 2004). They also release histolytic enzymes, reactive oxygen species and pro-inflammatory substances, which can lead to further inflammation and tissue damage (Hamada et al., 1996; Taoka et al., 1997; Xu et al., 1990).

Peripheral macrophages and activated microglia begin to infiltrate the injured spinal cord within 24 hours and peak infiltration occurs between 3 and 7 days (Blight, 1992; Carlson et al., 1998; Popovich, Wei, & Stokes, 1997). Lymphocytes begin to appear within 3 days and peak infiltration generally occurs at 7 days postinjury (Popovich et al., 1997). The emergence of immune cells at the injury site is, not surprisingly, accompanied by an increase in the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). In general, these cytokines are upregulated in the spinal cord within 6 hours after a contusion injury and return to normal levels within 48 hours (Nakamura et al., 2003; Nesic et al., 2001; Pineau & LaCroix, 2007; Wang, Olschowka, & Wrathall, 1997; Wang et al., 2005; Yang et al., 2005).

Role of Stress

Trauma to other parts of the body, especially the limbs, often accompany spinal cord injury and provide a source of nociceptive input to the spinal cord. This input acts as a physiological stressor and, depending on injury severity, may also serve as a psychological form of stress if it is perceived as painful. Physiological and psychological stressors initiate a cascade of events collectively known as the "stress response" that begins with the activation of the hypothalamic-pituitary-adrenal (HPA) axis and overall sympathetic arousal, and culminates in the release of glucocorticoids into the bloodstream. At low levels, such as that observed during mildly stressful events, glucocorticoids facilitate learning and memory (McEwen & Sapolsky, 1995). However, higher concentrations, induced by severe or chronic stressors, have detrimental effects

on these processes. This presumably reflects a glucocorticoid-mediated loss of neurons in areas of the brain important for learning and memory, such as the hippocampus (McEwen & Sapolsky, 1995). Interestingly, the impact of stress on the secondary damage that occurs after spinal cord injury has received very little attention.

We have shown that uncontrollable shock, which is routinely used in animal models of stress, impairs locomotor recovery, delays recovery of bladder function, increases mortality and spasticity, and exacerbates tissue loss after spinal cord contusion injury (Grau et al., 2004). Preliminary data suggest that these effects are related to a stress-induced release of corticosterone. For example, we have shown that uncontrollable shock causes an elevation of corticosterone in rats with a spinal cord injury that persists for up to 72 hours (Washburn et al., 2006). This result complements earlier findings that uncontrollable shock increases corticosterone in intact rats (Maier et al., 1986). Increases in corticosterone have been shown to potentiate cell loss in the CNS (Armanini et al., 1990; Chou, 1998). Although the mechanism underlying this glucocorticoid-mediated cell loss remains unknown, it appears that pro-inflammatory cytokines may play a role. For example, increases in pro-inflammatory cytokines are often correlated with increases in corticosterone (Turnbull et al., 1994; Zhou et al., 1993) and cell loss after injury (Nesic et al., 2001). Many types of stressors, including uncontrollable shock, have been shown to elevate pro-inflammatory cytokines (LeMay, Vander, & Kluger, 1990; Nguyen et al., 1998; O'Connor et al., 2003). Exposure to footshocks causes an increase in IL-1β in the brain as well as peripheral increases of IL-β (Maier & Watkins, 1998; Nguyen et al., 2000; O'Connor et al., 2003). The release of

IL-β is believed to activate macrophages and enhance leukocyte migration to areas of injury (Griffis, Compton, & Doering, 2006). The increase in corticosterone observed in spinal cord injured rats after exposure to uncontrollable shock may be accompanied by an increase in pro-inflammatory cytokines, which in turn may contribute to cell loss and impaired recovery of function.

Uncontrollable shock consists of two components that can induce a stress response. The first is the physiological component, which consists of the nociceptive input itself. The second is the psychological component, which consists of the perception of pain caused by the nociceptive input. The relative contribution of these two components to the consequences of uncontrollable shock remains unknown. In contused subjects, who retain some sensory function, these components must be separated. Morphine is often used to prevent the perception of pain and therefore can be used to block the psychological component. We have previously shown that morphine has no impact on the consequences of uncontrollable shock after contusion injury (Hook et al., 2007). This suggests that the nociceptive input itself plays a greater role than the psychological experience of pain in the behavioral outcome of uncontrollable shock. However, it is not known whether morphine treatment has any impact on the biochemical consequences of shock. If corticosterone is a key player, as suggested by preliminary data, then morphine treatment during shock exposure should have no effect on corticosterone levels or pro-inflammatory cytokine expression. It is possible that morphine may potentiate the biological consequences of uncontrollable shock given that others have shown that morphine increases plasma levels of corticosterone

(Budziszewska et al., 1999; Laorden & Milanes, 1999; Mellon & Bayer, 2001; Milanes, Puig, & Vargas, 1993; Simon, George, & Garcia, 1975) and elevates circulating levels of IL-1β and IL-6 (Bertolucci, Perego, & Simoni, 1996; Johnston et al., 2004; Houghtling & Bayer, 2002; Houghtling et al., 2000).

Another factor that may influence both the behavioral and the biochemical properties of shock exposure is the degree of controllability. Uncontrollable shock leads to a phenomenon known as "learned helplessness", in which subjects exhibit a behavioral, cognitive, and emotional deficit following exposure to inescapable, uncontrollable shock (Seligman, 1975). Subjects exposed to the same duration and frequency of controllable shock do not develop "learned helplessness", suggesting that it is not the shock *per se* that causes the behavioral, cognitive, and emotion deficits but rather the degree of perceived controllability. A number of biochemical alterations have been implicated in the development of "learned helplessness", including dysregulation of the noradrenergic system. Uncontrollable shock leads to a transient increase in norepinephrine followed by a decrease in norepinephrine in the locus coeruleus, hypothalamus, and hippocampus that persists for 24 hours.

Previous work has shown that controllability eliminates the consequences of shock after contusion injury. Subjects given the opportunity to control shock exposure did not show any locomotor impairment relative to unshocked controls (Grau et al., 2004). If corticosterone is involved in the behavioral consequences of uncontrollable shock, then we would expect subjects given control to have corticosterone levels comparable to unshocked subjects. Interestingly, others have shown that controllability

does not prevent the increase in corticosterone levels seen after shock exposure (Maier et al., 1986). This suggests that increases in corticosterone *per se* do not impact locomotor recovery. However, uncontrollable shock may cause a dysregulation of corticosterone. Our finding that corticosterone stays elevated in subjects that received uncontrollable shock for up to 72 hours supports this hypothesis (Washburn et al., 2006).

Specific Aims

The following set of experiments was designed to elucidate the mechanisms underlying the behavioral consequences of uncontrollable shock. It was divided into 4 experiments that focus on the major aspects of stress after injury. Table 1 outlines these experiments. The first experiment characterized the impact of the contusion injury itself on biological outcomes. It focused on some of the major players implicated in the consequences of stress: corticosterone, norepinephrine, and pro-inflammatory cytokines. We predict that the contusion injury will act as a stressor and produce a stress response characterized by increases in corticosterone and norepinephrine. Others have shown that the injury itself causes an increase in corticosterone (Popovich et al., 2001). They also showed that it causes an increase in pro-inflammatory cytokines at the injury site (Nakamura et al., 2003; Nesic et al., 2001; Pineau & LaCroix, 2007; Wang et al., 1997; Wang et al., 2005; Yang et al., 2005). We expect similar findings. We also expected that contusion injury would lead to a decrease in spleen weight given that other stressors are known to have this effect (Sumova & Jakoubek, 1989; Yamamotova et al., 2000).

Table 1. Outline of the experiments, including behavioral treatment, timepoint(s) and outcome measures of each.

Experiment 1: Impact of Contusion Injury on Biological Outcomes	Experiment 2: Impact of Uncontrollable Shock on Biological Outcomes after SCI Across Time	Experiment 3: Impact of Morphine Treatment During Uncontrollable Shock on Biological Outcomes after SCI	Experiment 4: Impact of Controllability on Biological Outcomes after SCI
Behavioral Treatment Anesthesia Alone Laminectomy Contusion Injury **Timepoint(s)** 24 hr	**Behavioral Treatment** Contusion Injury Unshocked Contusion Injury Shocked **Timepoint(s)** 6, 24, 72 hr and 7 d	**Behavioral Treatment** Laminectomy Morphine Unshocked Laminectomy Morphine Shocked Contusion Injury Morphine Unshocked Contusion Injury Morphine Shocked **Timepoint(s)** 24 hr	**Behavioral Treatment** Master Yoked Unshocked **Timepoint(s)** 24 hr
Outcomes BBB locomotor score Spleen weight Corticosterone Norepinephrine IL-β & IL-6	**Outcomes** BBB locomotor score Spleen weight Corticosterone Norepinephrine IL-β & IL-6	**Outcomes** BBB locomotor score Spleen weight Corticosterone Norepinephrine IL-β & IL-6	**Outcomes** BBB locomotor score Spleen weight Corticosterone Norepinephrine IL-β & IL-6 Immune cells at injury site Electrolytes

The second experiment assessed the impact of uncontrollable shock on these same biological outcomes over time. We have previously reported that subjects exposed to uncontrollable shock have diminished recovery of function and greater lesions at the injury site compared to unshocked controls (Grau et al., 2004). In that study, locomotor recovery was assessed over the course of 6 weeks and histological analysis occurred shortly thereafter. Although our basic analysis of tissue sparing at the lesion site provided an explanation for the diminished recovery, it did not address how uncontrollable shock could cause cell loss. Experiment 2 of the current study was designed to address this issue. We predict that uncontrollable shock will induce a stress response, resulting in increases in corticosterone and norepinephrine and a decrease in spleen weight. We also expect to find an increase in pro-inflammatory cytokines that could contribute to cell death and loss of function.

The third experiment used morphine treatment to identify the relative contribution of the physiological and psychological components to the biological consequences of uncontrollable shock exposure. Given our previous findings, we predict that morphine will have no effect on the consequences of uncontrollable shock. Morphine may even potentiate these effects.

The last experiment evaluated the effect of stressor controllability on corticosterone, norepinephrine, spleen weight, pro-inflammatory cytokine expression, and immune cell populations following spinal cord injury. Subjects given control over the shock should look comparable to unshocked controls. However, it is possible that shock, independent of the degree of controllability, will affect some of the biological outcomes measured.

CHAPTER II

GENERAL METHOD

Subjects

Male, Sprague-Dawley rats obtained from Harlan (Houston, TX) served as subjects. Animals were approximately 100-120 days old and weighed between 300 and 350 grams. Subjects were maintained on a 12-hour light-dark schedule and were housed individually. Food and water was available *ad libitum*. Behavioral testing was performed during the light portion of the cycle.

Surgery

Subjects received a contusion injury using the MASCIS device developed by Gruner (1992) and Constantini and Young (1994). Subjects were anesthetized with isoflurane (2%). Ten minutes later, spinal reflexes were assessed to verify that a stable level of anesthesia had been achieved. An area extending approximately 4.5 cm above and below the injury site was shaved and disinfected with iodine. A 7.0 cm incision was made over the vertebral column. Next, two incisions were made on either side of the vertebral column, extending about 3.0 cm rostral and caudal to the T10-T11 segment. The vertebrae dorsal and medial to T10-T11 were then cleared and the spinal tissue exposed. The vertebral column was fixed within the MASCIS device and a moderate injury was produced by allowing the 10-g impactor (outfitted with a 3.0 mm tip) to drop 12.5 mm. After injury, subjects were removed from the device, placed on a heating pad, and the wound was closed with Michel clips. To help prevent infection, subjects were treated with 100,000 units/kg Pfizerpen (penicillin G potassium) immediately after

surgery. For the first 24 hours after surgery, rats were placed in a recovery room maintained at 26.6°C. To compensate for fluid loss, subjects received 2.5 mL of saline after surgery.

Behavioral Procedures

Locomotor Recovery. Locomotor recovery was assessed using the Basso, Beattie, Bresnahan (BBB) locomotor recovery scale (Basso, Beattie, Bresnahan, 1995). All subjects were acclimated to the open-field testing apparatus (child's swimming pool) for 5 minutes, 3 days prior to surgery to prevent freezing behavior that often results from a novel environment. Care was taken to ensure that all investigators that scored locomotor recovery had both high intra- and inter-rater reliability and were blind to the treatment conditions. The first test session occurred 24 hours after surgery, prior to any experimental treatment. Subsequent test sessions occurred daily. Scores were transformed according to the procedure described in Ferguson et al., 2004.

Uncontrollable Tailshock. Subjects were loosely restrained in Plexiglas tubes as previously described in Crown et al., 2002. Intermittent constant current shock was applied through electrodes taped to the tail. Shocked rats received 80 ms tailshocks on a variable time schedule with a mean of 2 seconds (range = 0.2-3.8 s) for six minutes. Unshocked controls were placed in the restraining tubes, had the electrodes attached, but did not receive shock.

Master/Yoke Paradigm. Prior to training, the rat's rear legs were shaved, marked for placement of the shock leads, and a stainless steel wire was inserted over the tibia. The subject was then placed in the test apparatus and secured by means of a wire belt.

The contact electrode used to monitor leg position was taped to the plantar surface of the rat's foot using approximately 8-cm of porous tape (Orthaletic, 1.3 cm) with the end positioned immediately distal to the plantar protuberance. To minimize lateral leg movements, a piece of porous tape was wrapped around the leg above the ankle and taped to a bar extending across the apparatus directly under the front panel of restraining tube. The tape was adjusted so that it was taut enough to extend the knee, minimizing variability in leg position while not interfering with the flexion response. Next, a stainless steel wire was inserted into the tibialis muscle, 1.7 cm above the first electrode, and shock intensity adjusted to produce a 0.4 N flexion force (Grau, Barstow, & Joynes, 1998). The container of salt solution, which measures 18 cm long x 10 cm wide x 5.5 cm deep, was placed under the contact electrode and the level of the solution was adjusted so that the tip of the electrode was submerged by 4 mm. A third of the subjects (Master) then received 30minute of training with controllable shock. For subjects in this condition, shock only occurred when the contact electrode touched the underlying salt solution. A second group of subjects were experimentally yoked to the Master group. Each yoked rat was coupled to a master subject and received legshock at the same time, and for the same duration, as its master partner. For the yoked subjects, shock was uncontrollable—it occurred independent of leg position. A third group of subjects remained unshocked.

Master rats sometimes rapidly learn the instrumental requirement and, as a result, receive relatively little shock exposure. This could undermine both the potential benefit of instrumental training and the negative consequences of uncontrollable shock. To

avoid this problem, the performance of the master subjects was closely monitored. If a master rat successfully performed the instrumental response, and received no shock, for a period of 2 minutes during the first 10 minutes of testing, the response criteria was increased by raising the water level. Solution was added in 25 mL increments until it touched the contact electrode. This raised the water level by 4 mm. A second day of instrumental training (on the contralateral leg) was conducted after locomotor behavior was scored on Day 2. Subjects were prepared as described above and master rats received an additional 30 minutes of training. Yoked rats were again experimentally coupled to their master partners and received shock independent of leg position. Unshocked controls were set-up in the same fashion, but received no shock after the contact electrode depth was adjusted.

Biological Assays

Blood Collection and Preparation. Blood was drawn from the saphenous vein into a heparinized microcentrifuge tube immediately following shock treatment in all experiments. The samples were spun at 3000 x g for 15 minutes within 30 minutes of collection and the plasma was transferred into a clean microcentrifuge tube and stored at –20°C until further analysis. Trunk blood was collected through cardiac puncture at the time of sacrifice for all subjects. Samples were collected into heparinized blood collection tubes containing EDTA and were be spun at 3000 x g for 15 minutes within 30 minutes of collection. Plasma was aliquoted into clean microcentrifuge tubes and stored at –20°C until further analysis. Three mls of whole blood were reserved for white blood cell differential analysis and an electrolyte panel in Experiment 4.

Tissue Collection and Preparation. A 5 mm segment of spinal cord was collected from the injury site, snap frozen in liquid nitrogen, and stored at –80 °C until further analysis. At the time of analysis, the tissue was thawed in T-per (Pierce, Rockford, IL) containing protease inhibitor cocktail (Sigma, St. Louis, MO) and homogenized in a microcentrifuge tube using a pestle. Samples were then centrifuged at 4500 x *g* for 15 minutes at 4 °C and the supernatants were collected and stored at –80 °C. Total protein concentrations were assessed using a BCA Protein Assay Kit (Pierce, Rockford, IL) and spectrophotometer (Biomate 3, Thermo Electron Corporation, Waltham, MA) according to the manufacturers instructions. All tissue samples were diluted to a final total protein concentration of 500 μg/ml using T-per.

Corticosterone Assessment. Corticosterone levels were determined in all blood samples using an ELISA (Correlate-EIA kit, Assay Designs, Ann Arbor, MI, sensitivity = 26.99 pg/ml) following the manufacturer's instructions. Samples were read at a wavelength of 405 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA).

Norepinephrine Assessment. Norepinephrine levels were determined in trunk blood samples using an ELISA (Noradrenaline EIA, Rocky Mountain Diagnostics, Colorado Springs, CO, sensitivity = 44 pg/ml) following the manufacturer's instructions. Samples were read at a wavelength of 450 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA).

Pro-inflammatory Cytokine Analysis. The pro-inflammatory cytokines IL-1β and IL-6 were analyzed in tissue homogenates using ELISA kits (BioSource, Carlsbad, CA,

sensitivity < 3 and 8 pg/ml, respectively) according to the manufacturers instructions. Samples were read at a wavelength of 450 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA).

White Blood Cell Differential Analysis and Electrolyte Panel. A Celldyn 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL) was used to analyze lymphocytes and neutrophils in 1 ml of whole blood. Results were expressed as number of cells per ml of whole blood. Sodium, potassium, chloride, and carbon dioxide levels were also determined.

Data Analyses

Results were analyzed using an analysis of variance (ANOVA). In experiments with a temporal variable (e.g., exposure duration, recovery period), trend analyses were conducted to determine whether there is a significant linear (no inflection), quadratic (one inflection) or cubic (two inflections) trend. In cases where significant between subject differences were obtained, group means were compared using the Duncan's New Multiple Range Test. Locomotor scores were transformed to help assure that the data was amendable to parametric analyses (Ferguson et al., 2004). Additional statistical power was achieved by obtaining a measure of locomotor performance 24 hours after injury, prior to experimental treatment. This provided a behavioral index of the injury extent that correlated with long-term recovery (r 0.41, p 0.05; Hook et al., 2004). By using this factor as a covariate in an analysis of covariance (ANCOVA), we substantially reduce unexplained variance.

CHAPTER III

EXPERIMENT 1: IMPACT OF CONTUSION INJURY

Others have shown that both corticosterone and pro-inflammatory cytokines are increased following contusion injury (Popovich et al., 2001). The purpose of this experiment was to evaluate biological changes after contusion injury.

Method

Subjects (n=8) were assigned to one of three groups (anesthetized controls, sham, and contusion) in this experiment. Subjects received anesthesia alone, a laminectomy, or a contusion injury and locomotor ability was assessed using the BBB scale 24 hours later. Subjects were then restrained for 6 minutes. Blood was drawn from the saphenous vein immediately following restraint and subjects were returned to their home cages. Locomotor ability was re-assessed 24 hours later. Subjects were then euthanized with pentobarbital (100 mg/kg) and trunk blood was collected into a blood collection tube containing EDTA. Plasma corticosterone and norepinephrine were determined using ELISAs (Correlate-EIA kit, Assay Designs, Ann Arbor, MI, sensitivity = 26.99 pg/ml; Rocky Mountain Diagnostics, Colorado Springs, CO, sensitivity = 44 pg/ml, respectively). At the time of sacrifice, a 5 mm segment of spinal cord was taken at the injury site. The tissue was snap frozen in liquid nitrogen and stored at –80 °C until further analysis. Tissue was homogenized as described in the methods section and total protein concentration were assessed using a BCA Protein Assay Kit and spectrophotometer. All tissue samples were diluted with T-per as needed to obtain a final total protein concentration of 500 μg/ml. Tissue homogenates were assayed for the

pro-inflammatory cytokines IL-1β and IL-6 using ELISA kits (BioSource, Carlsbad, CA, sensitivity < 3 and 8 pg/ml, respectively) according to the manufacturers instructions. Spleens were also retained and weighed at the time of sacrifice because stress has been shown to impact spleen weights (Sumova & Jakoubek, 1989; Yamamotova et al., 2000).

Results

Locomotor Recovery. BBB locomotor scores, which are depicted in Figure 1, were recorded to ensure both adequate sham and contusion surgeries. Anesthetized controls and sham subjects had BBB scores of 12 (the highest converted score possible) at both times tested, demonstrating that neither laminectomy, restraint or blood collection affected locomotor ability. Subjects that received a contusion injury had scores of approximately 2.7 on both Day 1 and Day 2. An ANOVA on BBB scores from the last test session showed a significant effect of contusion injury, $F(2, 21) = 82.36$, $p < .05$. *Post hoc* comparisons of the group means revealed that only subjects that received a contusion injury differed from the other groups, $p < .05$. No other group differences were significant, $p > .05$.

We also performed analyses on the unconverted BBB scores. The reason for this is because the transformation proposed by Ferguson et al. (2004) is based on the assumption that subjects rarely exhibit BBB locomotor scores higher that 14. This is true for subjects that received a contusion injury but not the case for anesthetized controls and shams that exhibited scores of 20 and higher. Our analyses showed the exact same pattern of results. An ANOVA showed a significant effect of contusion injury, $F(2, 21) = 169.49$, $p < .05$. Again, *post hoc* comparisons of the group means

showed that only subjects that received a contusion injury differed from the other groups, $p < .05$. No other group differences were significant, $p > .05$.

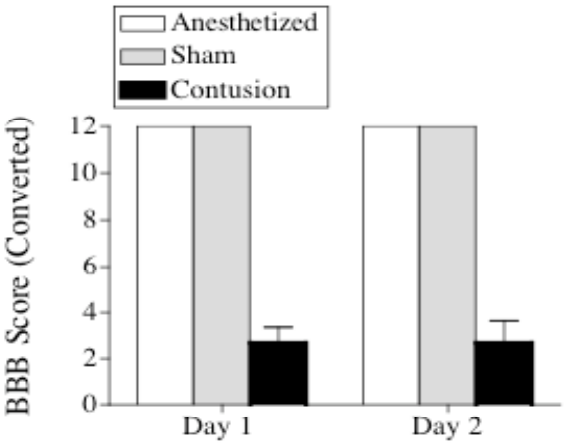

Figure 1. The impact of receiving anesthesia alone, a laminectomy, or a contusion injury on BBB locomotor scores.

Spleen Weights. Figure 2 shows spleen weights across the three conditions. To control for any differences in body weight, subject's weight at the time of sacrifice (last day weight) was used as a covariate in this analysis. An ANCOVA confirmed that there were no group differences for spleen weights, $F(2, 20) < 1.0$, $p > .05$.

Corticosterone ELISA. Corticosterone levels are shown in Figure 3. Corticosterone in blood taken from the saphenous vein (leg blood) immediately after restraint did not significantly vary across groups, $F(2,21) = 2.47$, $p > .05$. There was also no significant difference in corticosterone levels in blood collected at the time of sacrifice (trunk blood), $F(2,21) = 1.06$, $p > .05$.

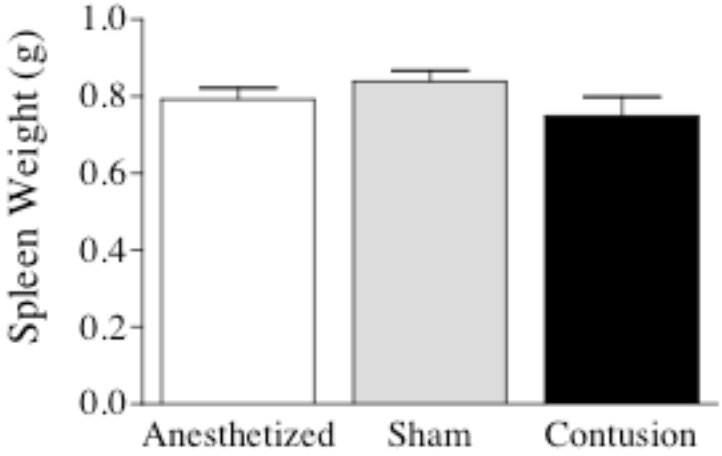

Figure 2. Spleen weights of subjects that received anesthesia alone, a laminectomy, or a contusion injury.

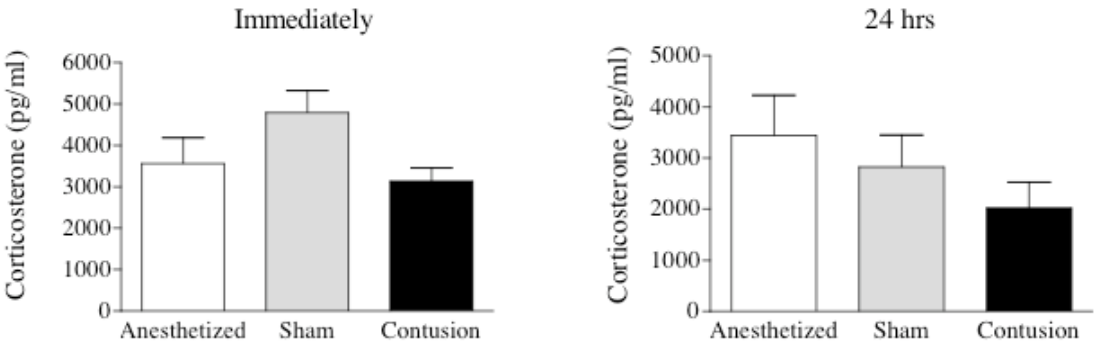

Figure 3. Effects of anesthesia, a laminectomy, or a contusion injury on plasma corticosterone levels immediately after restraint (left panel) and 24 hours after restraint (right panel).

Norepinephrine ELISA. Figure 4 shows norepinephrine levels. Analysis of norepinephrine levels in trunk blood also failed to produce any significant effects, $F(2, 21) = 1.29$, $p > .05$.

Pro-inflammatory Cytokine Analysis. Results from the analyses of IL-1β and IL-6 are illustrated in Figure 5. An ANOVA showed that neither IL-1β nor IL-6 concentrations at the injury site varied across groups, both Fs < 1.42, $p > .05$.

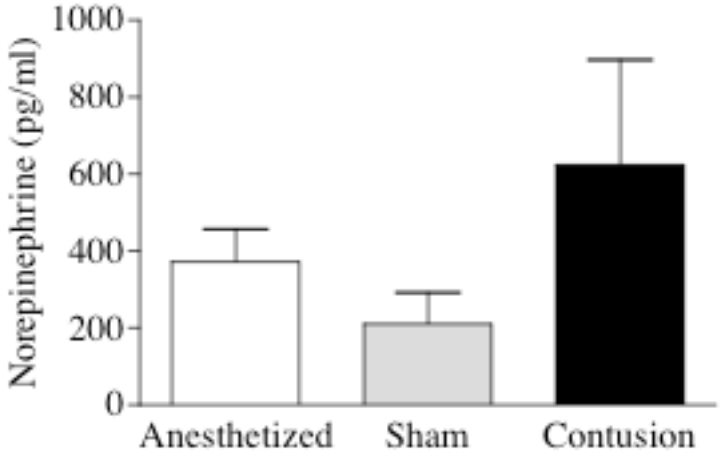

Figure 4. Plasma norepinephrine levels in subjects that received anesthesia alone, a laminectomy, or a contusion injury.

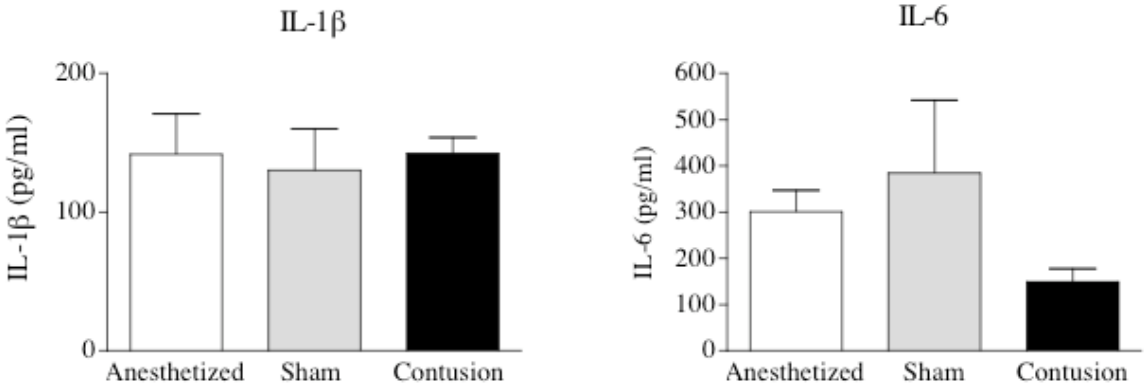

Figure 5. Impact of anesthesia alone, a laminectomy, or a contusion injury on IL-1β and IL-6 protein levels at the injury site.

Discussion

Experiment 1 showed that contusion injury has no impact on any of the biological outcomes assessed at 24 hours, including corticosterone, norepinephrine, IL-1β, and IL-6 levels, and does not produce any significant changes in spleen weights.

CHAPTER IV

EXPERIMENT 2: IMPACT OF UNCONTROLLABLE SHOCK

Experiment 1 examined the impact of contusion injury on several common biological outcomes. The following experiment was designed to assess the impact of receiving uncontrollable shock following contusion injury on these same biological outcomes. These changes were initially assessed 24 hours following shock treatment but then three additional timepoints were added. Results from the 24 hour timepoint will be presented first, followed by results across the four timepoints.

Method

All subjects (n=8) received a contusion injury and locomotor ability was assessed using the BBB scale 24 hours later. Subjects then received 6 minutes of uncontrollable tailshock or an equivalent amount of tube restraint. Blood was drawn from the saphenous vein immediately following shock treatment and subjects were returned to their home cages. Locomotor recovery was assessed once daily thereafter until the time of sacrifice, 6, 24, 72, or 168 hours later. On the final day of locomotor testing, subjects were euthanized with pentobarbital (100 mg/kg) and blood and spinal cord tissue was collected. Corticosterone, norepinephrine, and pro-inflammatory cytokine levels were then assessed using the procedures previously described in Experiment 1. Spleens were also retained and weighed at the time of sacrifice.

Results from the 24 hour Timepoint

Locomotor Recovery. All results from the 24 hour timepoint are depicted in Figure 6. BBB locomotor recovery scores are shown in Figure 6A. BBB scores were

taken prior to any experimental treatment to ensure no pre-existing group differences. An ANOVA on Day 1 BBB scores confirmed that the groups did not differ, $F(1, 14) < 1.0$, $p > .05$. On the last test day, unshocked subjects tended to exhibit higher BBB scores. However, an ANCOVA, using Day 1 BBB as a covariate, failed to show a significant effect of shock, $F(1, 13) = 2.11$, $p > .05$.

Spleen Weights. Last day weight was used as a covariate in spleen weight analysis to control for any variance in body weight. As can be seen in Figure 6B, uncontrollable shock caused a decrease in spleen weight. An ANCOVA confirmed a significant effect of shock, $F(1, 13) = 6.11$, $p < .05$.

Corticosterone ELISA. Results from the corticosterone ELISA can be seen in Figure 6C. An ANOVA on corticosterone levels in blood taken immediately after shock treatment failed to show any significant group differences, $F(1, 14) < 1.0$, $p > .05$. At 24 hours after shock treatment, shocked subjects exhibited higher level of corticosterone, but this effect did not reach statistical significance, $F(1, 14) = 3.51$, $p > .05$.

Norepinephrine ELISA. Figure 6D show the results from the norepinephrine ELISA. An ANOVA showed no significant group differences on this measure, $F(1, 14) = 1.17$, $p > .05$.

Pro-inflammatory Cytokine Analysis. IL-1β and IL-6 results can be seen in Figures 6E and F, respectively. Uncontrollable shock caused a significant increase in IL-1β at the 24 hour timepoint, $F(1, 14) = 5.59$, $p < .05$ but had no effect on IL-6, $F(1, 14) < 1.0$, $p > .05$.

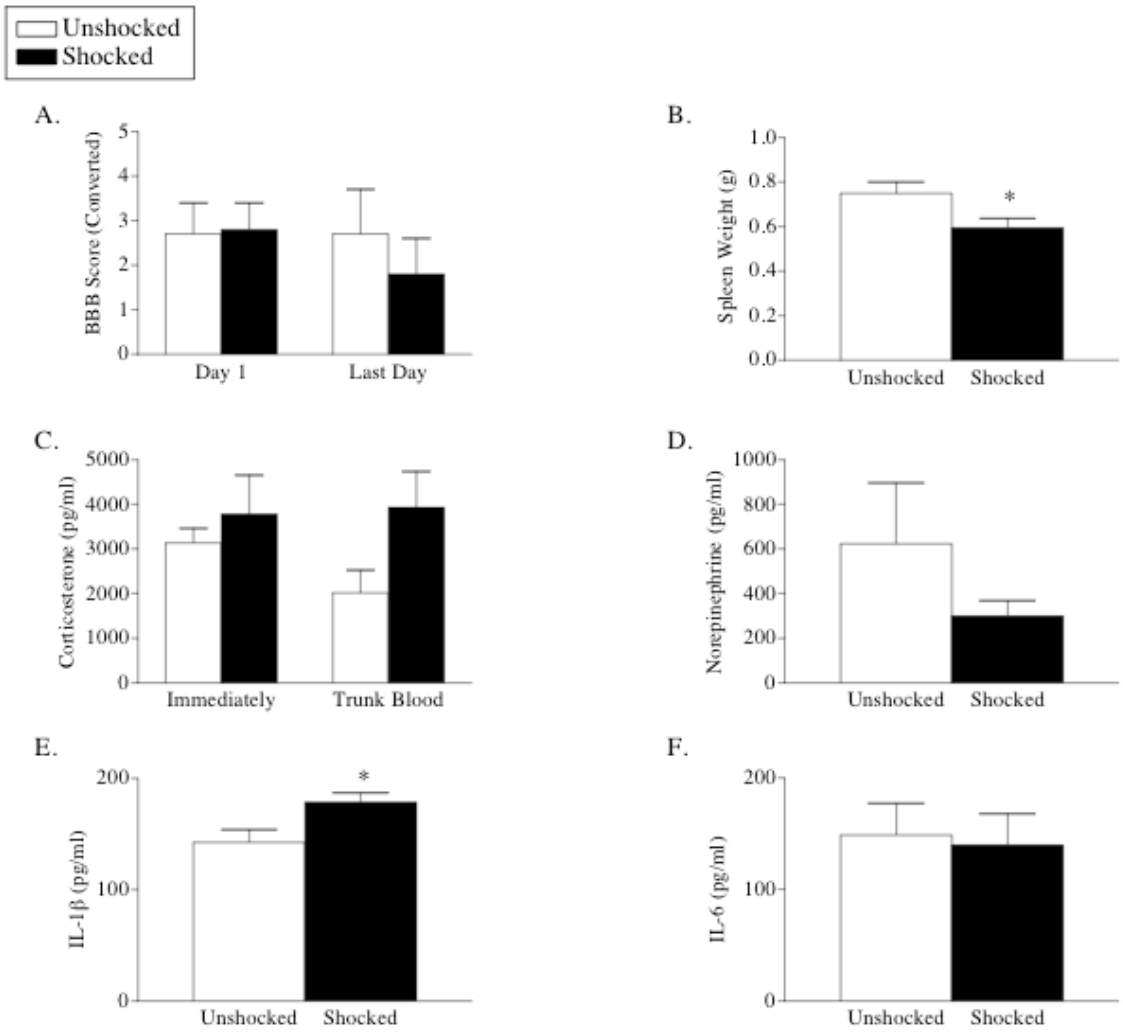

Figure 6. Effects of uncontrollable shock at 24 hours on BBB (A), spleen weight (B), plasma corticosterone (C), plasma norepinephrine (D), and IL-1β (E) and IL-6 (F) protein content at the injury site.

Results Across Time

Locomotor Recovery. BBB scores, which were taken prior to any experimental treatment to ensure no pre-existing group differences, ranged from 3.56 (± 0.89) to 5.00 (± 0.91). An ANOVA on Day 1 BBB scores confirmed no pre-existing group differences, Fs < 1.0, p > .05. Figure 7 illustrates BBB scores from the last test sessions across the four timepoints. As expected, all subjects showed an increase in BBB score across time. However, scores from the last test session were higher in the unshocked subjects at all timepoints assessed. An ANCOVA on last test session scores using Day 1 BBB scores as a covariate revealed significant main effects of shock and time, Fs > 12.27, p < .05. *Post hoc* comparisons of the group means showed that unshocked subjects differed significantly from shocked subjects at the 72 and 168 hour timepoints, p < .05.

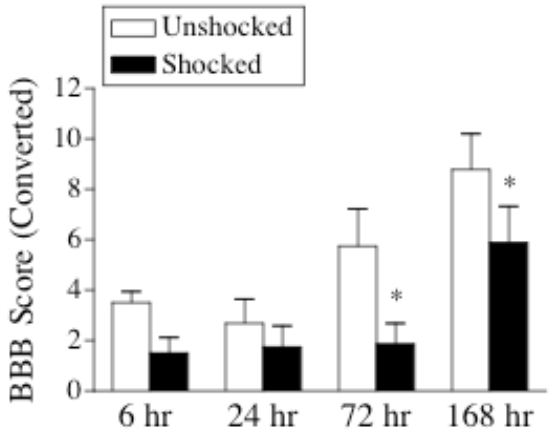

Figure 7. Impact of uncontrollable shock on locomotor ability across time.

Spleen Weights. Again to control for any variance in body weight, last day weight was used as a covariate in spleen weight analysis. Overall, spleen weights from shocked subjects were significantly lower than those of unshocked controls. An ANCOVA verified significant main effects of shock and time, Fs > 12.48, $p < .05$. *Post hoc* comparisons of the group means, which are displayed in Figure 8, showed that spleen weights were significantly lower in shocked animals at the 24, 72, and 168 hour timepoints, $p < .05$.

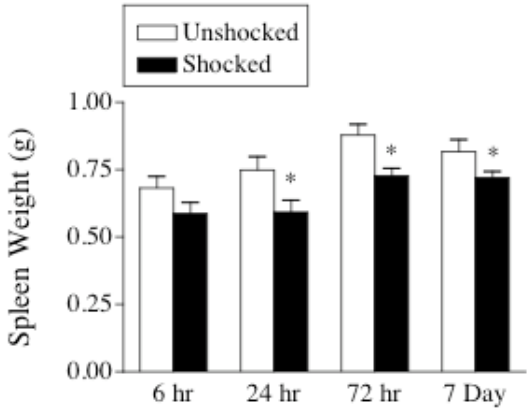

Figure 8 The effects of uncontrollable shock on spleen weight in subjects sacrificed 6, 24, 72, and 168 hrs after shock treatment.

Corticosterone ELISA. Because blood was collected from the leg immediately after shock treatment in all subjects regardless of sacrifice timepoint, results from this analysis were collapsed across the first three timepoints (Figure 9A). There was not a significant difference in corticosterone levels between unshocked and shocked subjects in blood taken from the leg immediately after shock treatment, $F(1, 46) < 1.0$, $p > .05$. In trunk blood, corticosterone levels were elevated in both shocked and unshocked subjects

at the 6 hour timepoint. *Post hoc* comparisons of the group means showed that these levels were significantly decreased in unshocked animals at all other timepoints, $p < .05$. However, corticosterone levels remained elevated in the shocked subjects at all timepoints tested (Figure 9B). An ANOVA confirmed significant main effects of shock and time, Fs > 4.07, $p < .05$.

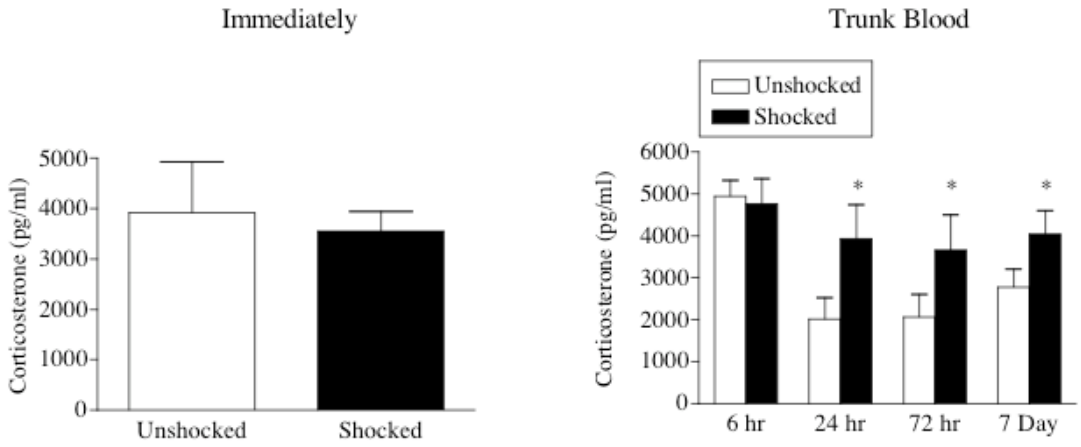

Figure 9. Plasma corticosterone levels in shocked and unshocked subjects. The left panel depicts plasma levels immediately after shock treatment. The right panel shows plasma corticosterone levels at the time of sacrifice.

Norepinephrine ELISA. Norepinephrine results are depicted in Figure 10. An ANOVA failed to show any significant differences in norepinephrine levels in the blood between shocked and unshocked subjects at any of the timepoints tested, Fs < 1.0, $p >$ .05.

Pro-inflammatory Cytokine Analysis. IL-1β and IL-6 results are shown in Figure 11. Overall, IL-1β levels decreased over the course of 168 hours. An ANOVA on results from the IL-1β ELISA revealed only a significant effect of time, $F(1, 3) = 5.36$, p

< .05. There was not a significant effect of uncontrollable shock on IL-1β levels at the injury site across time. For IL-6, uncontrollable shock caused a significant increase at the 6 hour timepoint. An ANOVA confirmed a significant main effect of time and a significant Shock X Time interaction, *F*s > 14.53, $p < .05$. Further analysis of the individual timepoints with a series of t-tests using a Bonferroni adjustment revealed that unshocked subjects differed significantly from shocked subjects only at the 6 hour timepoint, $t(56) = 5.89$, $p < .05$.

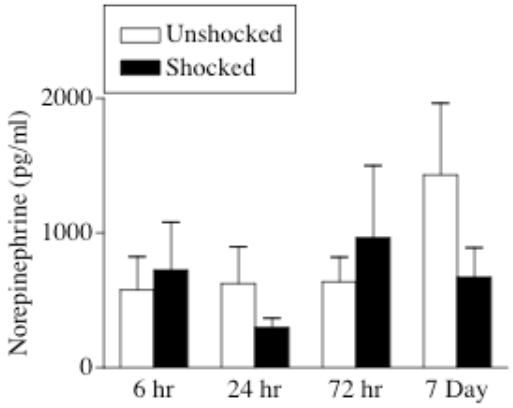

Figure 10 . Plasma norepinephrine concentrations in shocked and unshocked subjects across time.

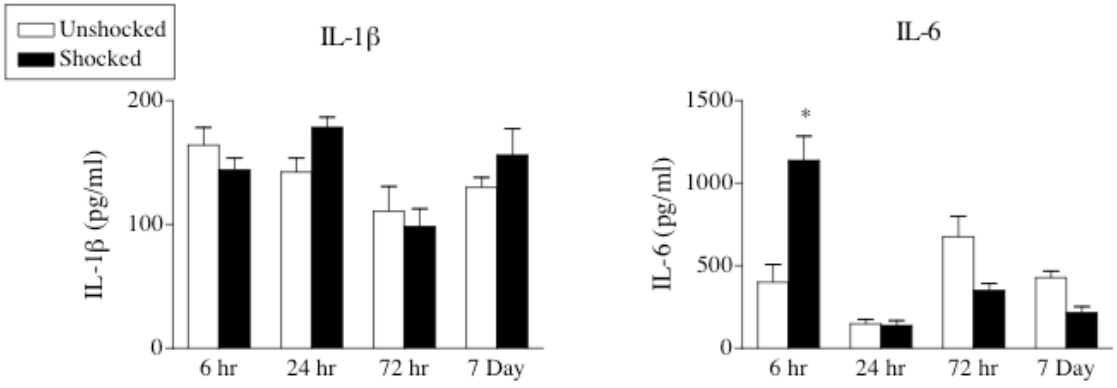

Figure 11. Impact of uncontrollable shock on IL-1β and IL-6 protein levels at the injury site across time.

Discussion

Uncontrollable shock caused a locomotor deficit that emerged within 72 hours and persisted for up to 168 hours. This locomotor deficit was accompanied by a decrease in spleen weight within 24 hours. This decrease in spleen weight also persisted for up to 168 hours. Uncontrollable shock also impacted many of the other biological outcomes tested. For example, both shocked and unshocked subjects had elevated levels of corticosterone in trunk blood 6 hours after treatment. These levels decreased in the unshocked subjects within 24 hours but remained elevated throughout the 168 hours in subjects that received shock. Shocked subjects also had higher levels of the pro-inflammatory cytokines IL-1β and IL-6 in tissue from the injury site.

CHAPTER V

EXPERIMENT 3: IMPACT OF MORPHINE

Uncontrollable shock is a stressor that consists of two components: a physiological component and a psychological component. The relative role of these two components in the detrimental effects of uncontrollable shock remains unknown. We have previously shown that morphine treatment, which blocks the psychological experience of pain, does not affect the behavioral consequences of uncontrollable shock. This suggests that the psychological component may play little role in our effect. The following experiments were designed to address this issue.

Method

Subjects (n=8) received either a contusion injury or laminectomy and locomotor ability was assessed using the BBB scale 24 hours later. Subjects then received morphine (20 mg/kg; i.p.) and were given 6minutes of uncontrollable tailshock or an equivalent amount of tube restraint thirty minutes later. Laminectomy subjects were used to assess the feasibility of using morphine during uncontrollable shock exposure in rats with an intact spinal cord. Our hope was that morphine would not interfere with the behavioral or the biochemical consequences of uncontrollable shock in these subjects so that it could be used to prevent the experience of pain in future experiments that require laminectomy controls. Blood was drawn from the saphenous vein immediately following shock treatment and subjects were returned to their home cages. Locomotor ability was re-assessed 24 hours later. Subjects were then euthanized with pentobarbital (100 mg/kg), blood and spinal cord tissue was collected and corticosterone,

norepinephrine, and pro-inflammatory cytokine levels were assessed using the procedures described in Experiment 1.

In order to compare the effect of morphine treatment versus no treatment on locomotor recovery and biological indices of stress, shocked and unshocked subjects from the 24 hour timepoint in Experiment 2 were used in all analyses. The procedure used in these subjects was identical to that used in the current experiment with the exception of morphine treatment.

Results

Locomotor Recovery. BBB scores for Days 1 and 2 are depicted in Figure 12. Figure 12A shows the scores for all subjects that received morphine. Figure 12B shows the scores for all subjects that received a contusion injury. An ANOVA confirmed a significant main effect of injury for subjects that received morphine, $F(1, 28) = 1.60$, $p < .05$. All subjects that received a laminectomy had BBB scores of 20 or higher, indicating that little to no spinal cord damaged occurred. For contused subjects, an ANOVA showed no group differences on Day 1 BBB scores for subjects that received contusion injury, Fs < 1.0, $p > .05$.

In morphine-treated subjects, an ANCOVA revealed significant main effects of injury and shock, Fs > 4.13, $p < .05$. *Post hoc* comparisons showed that uncontrollable shock only caused a significant decrease in BBB scores in contused subjects, $p < .05$. The fact that uncontrollable shock had no impact on locomotor scores in laminectomy subjects suggests that shock only produces detrimental effects in the presence of a contusion injury. In contused subjects, uncontrollable shock caused a significant

decrease in BBB scores in morphine-treated subjects and subjects that received no treatment. An ANCOVA using Day 1 BBB as a covariate confirmed a significant main effect of shock, $F(1, 27) = 4.60$, $p < .05$.

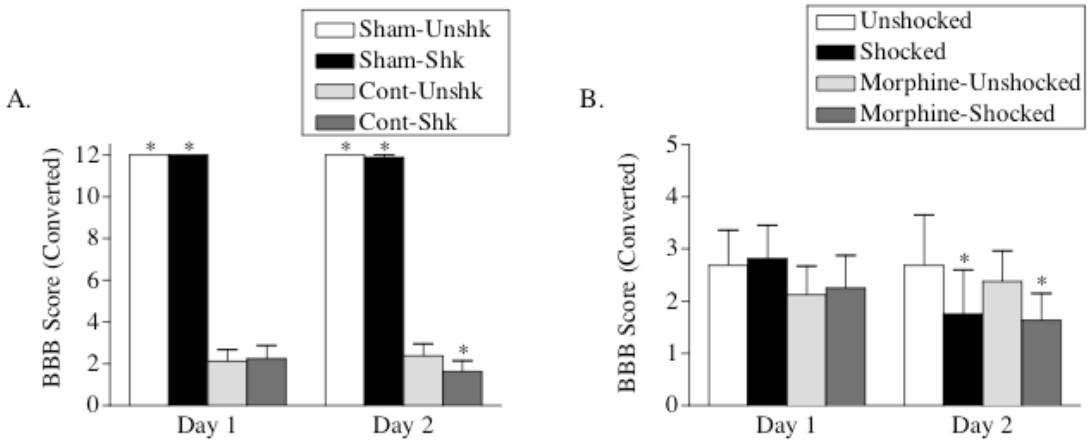

Figure 12. The effect of receiving morphine treatment during uncontrollable shock on BBB locomotor scores. Figure 12A shows the results from all morphine-treated subjects and Figure 12B depicts those from all contused subjects. The left panels show BBB scores prior to any experimental treatment. The right panels show BBB scores 24 hours after shock exposure.

Spleen Weights. Spleen weights are shown in Figure 13, with morphine-treated subjects in Figure 13A and contused subjects in Figure 13B. Subject's last day weights were again used as a covariate in all analyses. An ANCOVA on spleen weights from all morphine-treated subjects revealed no significant effects, Fs < 1.05, $p > .05$. In contused subjects, uncontrollable shock caused a decrease in spleen weights. An ANCOVA showed a significant main effect of shock, $F(1, 27) = 4.50$, $p < .05$. *Post hoc* analysis demonstrated that subjects that did not receive morphine and remained unshocked were significantly different than unshocked subjects that received morphine and shocked

subjects that did not receive morphine, $p < .05$. No other group differences were significant, $p > .05$.

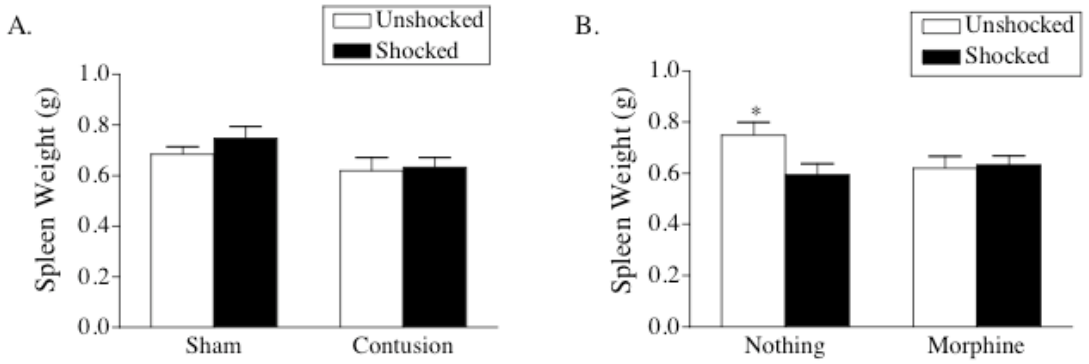

Figure 13. The impact of receiving morphine treatment during uncontrollable shock on spleen weight. Figure 13A shows the results from all morphine-treated subjects and Figure 13B depicts those from all contused subjects.

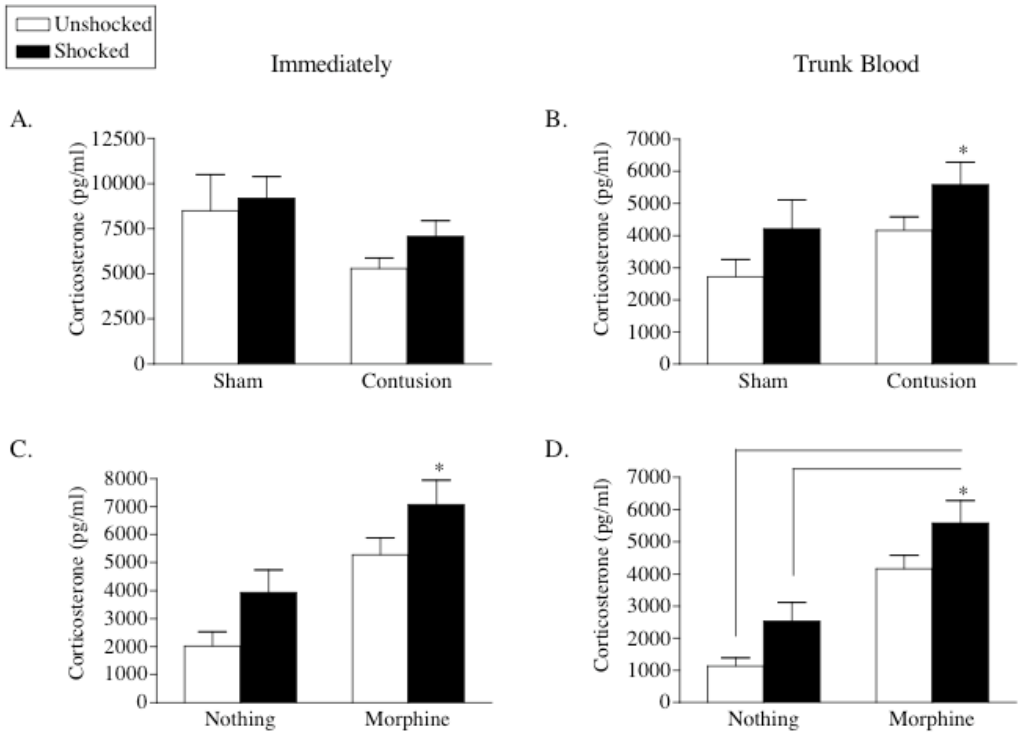

Figure 14. Plasma corticosterone levels in all morphine-treated (top graphs) and contused subjects (bottom graphs). Figures 14 A&C represent corticosterone levels immediately after shock treatment and Figures 14B&D show those 24 hours after shock.

Corticosterone ELISA. Figure 14 depicts the impact of shock and morphine treatment on corticosterone levels. Figure 14A shows the data for the blood collected from the leg immediately after shock treatment in all morphine-treated subjects and Figure 14C shows the same for all contused subjects. No significant effects were found for corticosterone levels in blood collected immediately after shock treatment in the comparison of all morphine-treated subjects, Fs < 3.80, $p > .05$. There was, however, a significant main effect of drug among contused subjects, $F(1,28) = 12.79$, $p < .05$. In general, morphine caused an increase in corticosterone. *Post hoc* analysis of the group means showed that morphine-treated subjects that received uncontrollable shock were significantly different than both saline groups, $p < .05$. No other group differences reached significance, $p > .05$.

Figure 14B illustrates the results of the corticosterone analysis from trunk blood in morphine-treated subjects. Figure 14D shows that for all the subjects that received a contusion injury. Uncontrollable shock caused an increase in corticosterone among all subjects that received morphine. A two-way ANOVA revealed only a significant main effect of shock, $F(1,28) = 4.18$, $p < .05$. *Post hoc* comparisons of the group means showed that contused subjects that received shock after morphine-treatment differed significantly from subjects that received a laminectomy and no shock after morphine treatment, $p < .05$. No other significant group differences were noted among all morphine-treated subjects, $p > .05$. In contused subjects, both uncontrollable shock and morphine caused an elevation in corticosterone. A two-way ANOVA showed significant main effects of both shock and drug, Fs > 6.42, $p < .05$. *Post hoc*

comparisons showed that both morphine groups (shocked and unshocked) had significantly higher corticosterone levels than both saline groups, $p < .05$. There were no other significant group differences, $p > .05$.

Norepinephrine ELISA. Norepinephrine levels are shown in Figure 15. Figure 15A illustrates those in all morphine-treated subjects and Figure 15B shows those for all contused subjects. No significant group differences were noted among either morphine-treated subjects, Fs < 2.60, $p > .05$ or contused subjects, Fs < 1.76, $p > .05$.

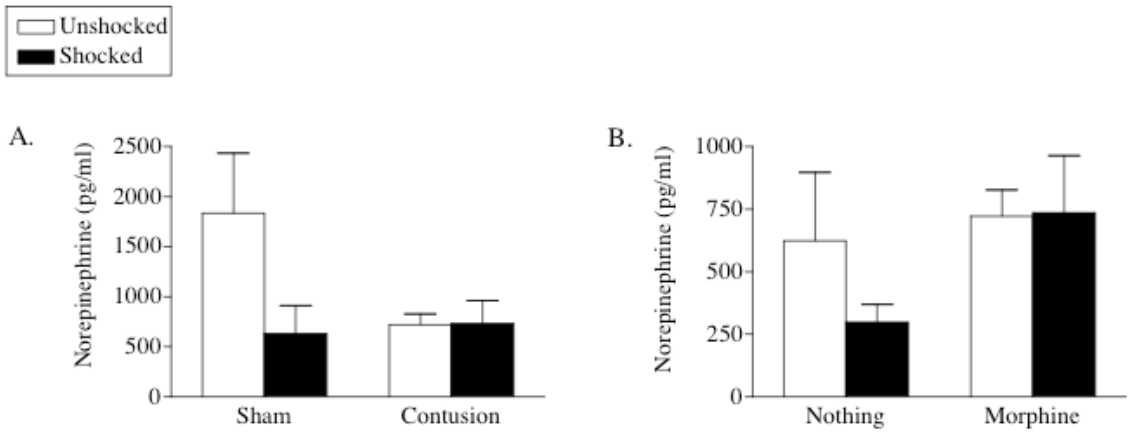

Figure 15. Norepinephrine levels in plasma for all morphine-treated (A) and all contused subjects (B).

Pro-inflammatory cytokine ELISAs. Figure 16 shows the results from the analyses of the pro-inflammatory cytokines IL-1β and IL-6 at the injury site in morphine-treated subjects (Figure 16A & C) and contused subjects (Figure 16B and D). Among morphine-treated subjects, contused subjects had lower levels of IL-1β than subjects that received only a laminectomy and this effect occurred independent of shock treatment. An ANOVA showed a significant main effect of injury, $F(1,28) = 35.20$, $p <$

.05. *Post hoc* comparisons showed that both contused subjects that did not receive shock and those that did receive shock differed significantly from both laminectomy groups, $p < .05$. No other comparisons reached significance, $p > .05$. Similar results were noted for IL-6 among morphine-treated subjects. Again, contused subjects had lower levels of IL-6 than subjects that received laminectomy alone. An ANOVA confirmed a significant main effect of injury, $F(1,28) = 6.76$, $p < .05$. *Post hoc* comparisons of the group means showed that subjects that received a laminectomy (shams) and shock were significantly different from both contusion groups, $p < .05$. No other group differences were significant, $p > .05$.

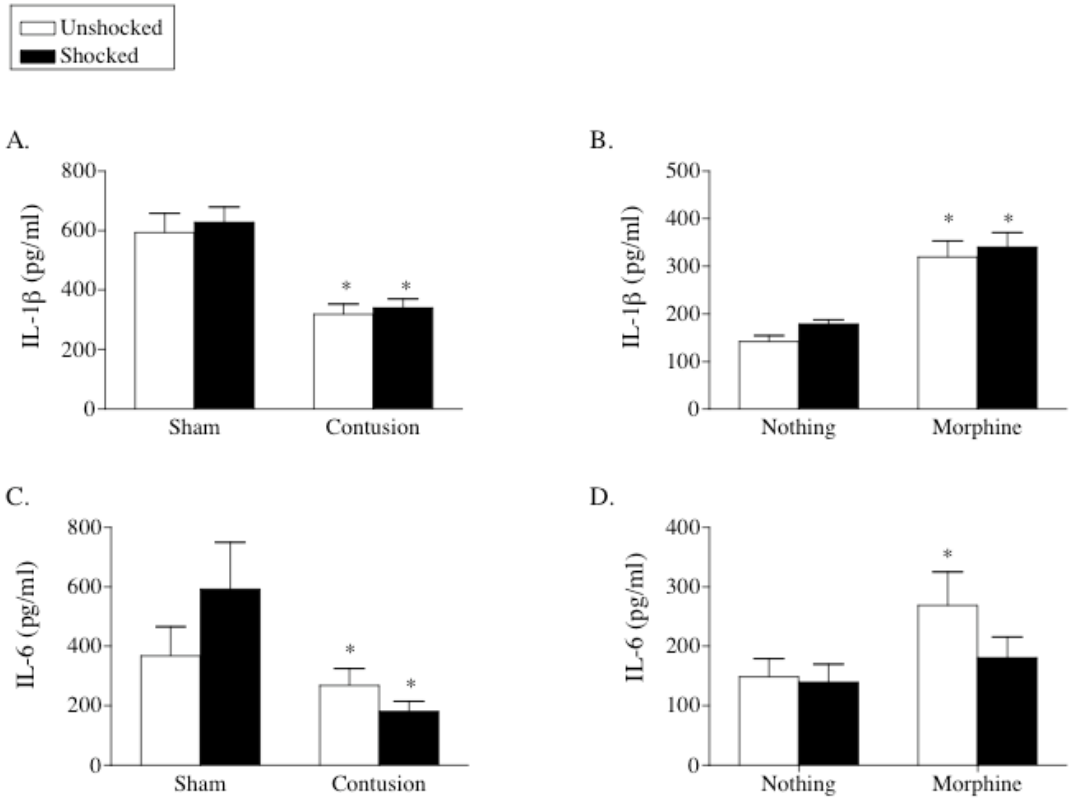

Figure 16. Impact of receiving morphine during uncontrollable shock on IL-1β and IL-6 protein levels at the injury site. Figures 16 A&C show the results for all morphine-treated subjects and Figures 16B&D show those for contused subjects.

In contused subjects, morphine caused a significant increase in IL-β independent of shock treatment. An ANOVA confirmed a significant main effect of drug, $F(1,28) = 48.57$, $p < .05$. *Post hoc* comparisons revealed that both morphine groups differed significantly from both saline groups, $p < .05$. No other significant group differences were found, $p > .05$. A similar pattern of results emerged for IL-6, with morphine causing an increase in IL-6 that was again independent of shock treatment. An ANOVA showed that there was a significant main effect of drug, $F(1,28) = 4.15$, $p < .05$. *Post hoc* analysis of the group means confirmed that unshocked subjects that received morphine had significantly higher IL-6 levels than shocked subjects that received no treatment, $p < .05$. No other group differences reached significance, $p > .05$.

Discussion

In the current experiment, uncontrollable shock produced a locomotor deficit only in contused subjects and morphine did not attenuate this effect. Several biological changes also occurred within 24 hours of shock treatment. Uncontrollable shock caused a decrease in spleen weights and increase in corticosterone in contused subjects. Again, morphine treatment failed to attenuate these effects. Instead, morphine often exacerbated or produced changes that were independent of shock treatment. For example, morphine caused dramatic increases of corticosterone and IL-1β and a significant increase of IL-6 in contused subjects.

CHAPTER VI

EXPERIMENT 4: IMPACT OF CONTROLLABILITY

Controllability has been shown to be a factor in whether a stressor has negative effects. For the most part, control is a good thing. We have shown that if subjects have control over shock exposure, shock has no effect on locomotor recovery. However, controllability typically does not prevent the biological consequences of exposure to shock, such as overall increases in corticosterone. The following experiments were designed to determine the impact of receiving controllable versus uncontrollable shock after spinal cord injury on corticosterone, norepinephrine, pro-inflammatory cytokines, and immune cell populations.

Methods

Subjects (n=8) received a contusion injury and locomotor ability was assessed using the BBB scale 24 hours later. Subjects were then exposed to the master/yoke paradigm as described in the methods. This involved two days of training. Locomotor ability was re-assessed 24 hours after the last training session and subjects were euthanized with pentobarbital (100 mg/kg). Blood and spinal cord tissue was collected and corticosterone, norepinephrine, and pro-inflammatory cytokine levels were assessed using the procedure described in Experiment 1. Approximately 2 ml of whole blood was retained from the trunk blood and white blood cell differential analysis and an electrolyte panel was performed as described in the methods.

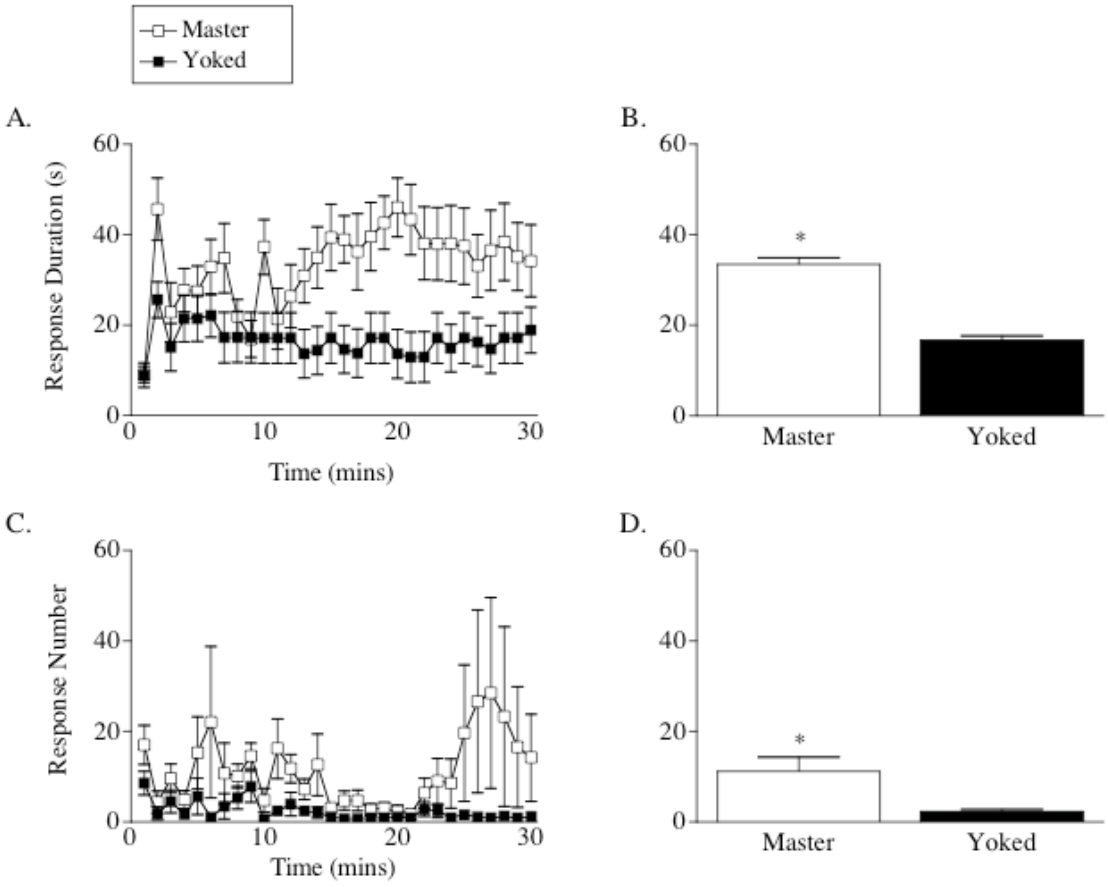

Figure 17. Response durations (A) and response numbers (B) during instrumental training with controllable (Master) and uncontrollable (Yoked) shock across time. The results from Days 1 & 2 of training have been collapsed. Mean response durations and response numbers are shown in the right panels (B & D, respectively).

Results

Instrumental Training. Analysis of response durations from Day 1 and Day 2 showed that there was not a significant difference across days, allowing us to collapse the two days for further analysis. Performance during the instrumental training procedure for master and yoked subjects is depicted in Figure 17. Response durations and response numbers from Days 1 and 2 have been collapsed across the two days.

Response duration and response number across time are illustrated in Figures 17A & C, respectively. Mean response duration and response number collapsed across time are shown in Figures 17B & D, respectively. An ANOVA showed that neither day nor its interaction with shock or session time approached significance, Fs < 3.37, $p > .05$. As expected, master subjects exhibited an increase in response duration, indicative of learning, over time, whereas yoked subjects did not. An ANOVA on mean response durations showed a significant difference between master and yoked subjects, $F(1,12) = 6.78$, $p < .05$. There was also a significant main effect of time and a Time X Group interaction, both Fs > 1.55, $p < .05$.

For response number, an ANOVA confirmed only a significant main effect of group, $F(1,12) = 6.78$, $p < .05$. On average, master subjects made more responses than their yoked partners. This resulted from yoked rats habituating to the shock and, therefore making fewer responses.

Locomotor Recovery. Figure 18 illustrates the impact of controllable versus uncontrollable shock on locomotor recovery. An ANOVA confirmed no pre-existing group differences on Day 1 BBB scores, $F(2,18) < 1.0$, $p > .05$. Master subjects that received controllable shock showed an increase in BBB scores over the two days of locomotor testing, whereas yoked and unshocked subjects did not. An ANCOVA using Day 1 BBB score as the covariate, showed a significant main effect of group, $F(2,17) = 3.48$, $p < .05$. *Post hoc* comparison of the group means showed that master subjects had significantly higher BBB scores than both yoked subjects and unshocked controls across the two days, $p < .05$.

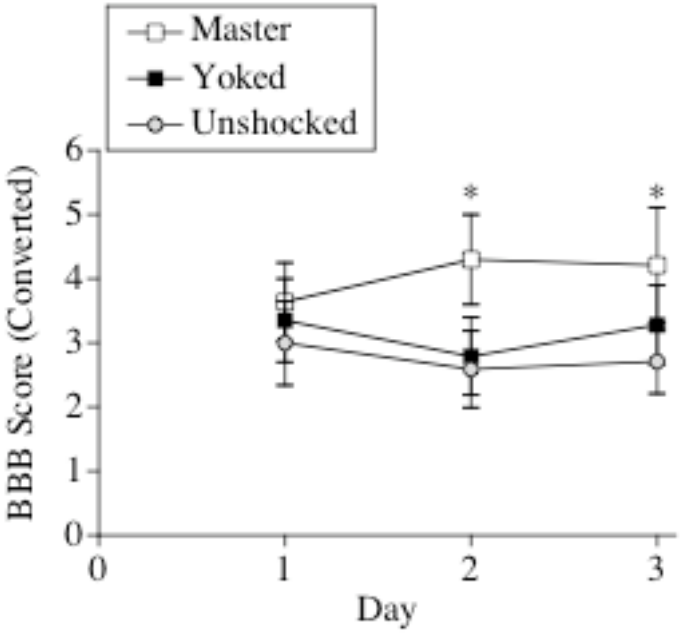

Figure 18. BBB locomotor scores from master, yoked, and unshocked subjects across the three days of testing.

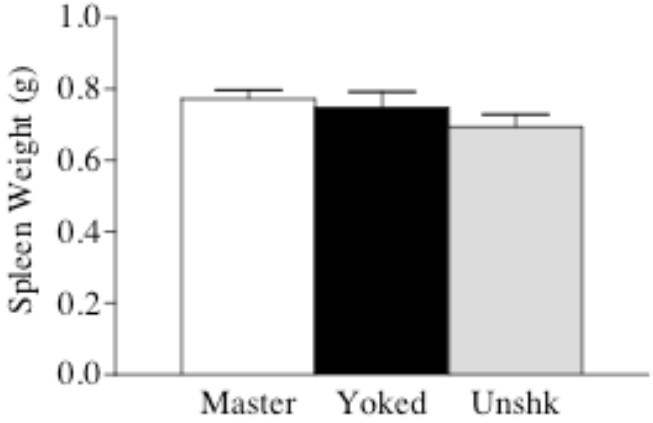

Figure 19. Impact of controllability on spleen weights 24 hours following the last instrumental training session.

Spleen Weights. Spleen weights across the three groups are shown in Figure 19.

No significant differences in spleen weights were found, $F(2,18) = 1.10$, $p > .05$.

Corticosterone ELISA. Figure 20 depicts corticosterone levels in trunk blood among master, yoked, and unshocked subjects. Interestingly, unshocked controls exhibited the highest level of corticosterone. An ANOVA confirmed a significant effect of group, $F(2,18) = 5.44$, $p < .05$. *Post hoc* analysis among the three groups showed that unshocked subjects had significantly higher corticosterone levels than both master and yoked subjects. This will be addressed later in the discussion section.

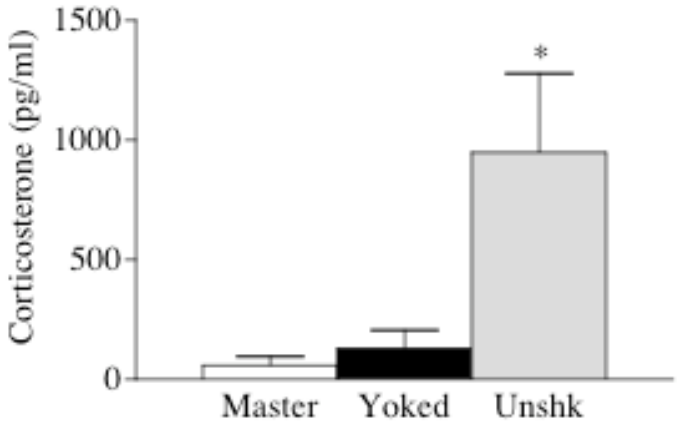

Figure 20. Plasma corticosterone levels in master, yoked, and unshocked subjects 24 hours after the last instrumental training session.

Norepinephrine ELISA. Figure 21 shows the impact of controllable versus uncontrollable shock on norepinephrine levels in trunk blood. Although master subjects appeared to have higher levels of norepinephrine, an ANOVA failed to find any significant effects, $F(2,18) = 1.62$, $p > .05$.

Pro-inflammatory Cytokine Analysis. The results from the IL-1β and IL-6 ELISAs are shown in Figure 22. There were no significant differences across groups for IL-1 β, $F(2,18) = 1.29$, $p > .05$. However, unshocked subjects had significantly higher

levels of IL-6 than both master and yoked subjects, $F(2,18) = 3.90$, $p < .05$. *Post hoc* analysis confirmed that this effect was significant, $p < .05$.

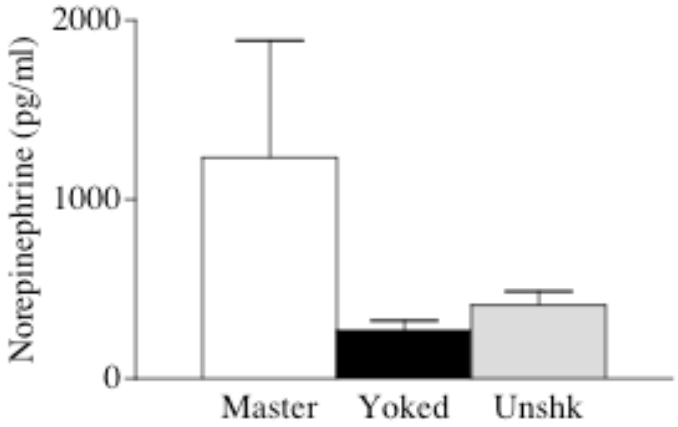

Figure 21. Impact of controllability on plasma norepinephrine levels 24 hours following the last instrumental training session.

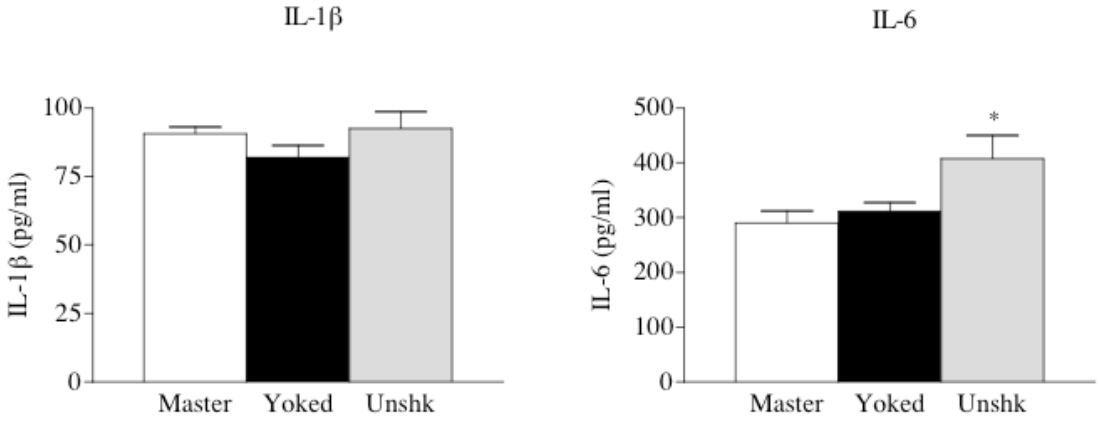

Figure 22. IL-1β and IL-6 protein levels at the injury site for master, yoked, and unshocked subjects. Protein content was analyzed 24 hours after the last training session.

White Blood Cell Differential Analysis. The impact of controllable and uncontrollable shock on the number of neutrophils, lymphocytes, monocytes and

eosinophils in the blood are depicted in Figure 23. No group differences were found for neutrophils, lymphocytes, or eosinophils, Fs < 1.31, $p > .05$. An ANOVA on blood monocytes showed a significant effect of shock, $F(2,18) = 3.63$, $p < .05$. *Post hoc* comparison of the group means showed that unshocked subjects had a significantly higher number of monocytes than master subjects. The reasons for this are unknown.

Electrolyte Panel. The results from the electrolyte panel are displayed in Figure 24. Sodium, potassium, chloride, and carbon dioxide levels were assessed. No group differences were noted for any of these electrolytes, Fs < 1.38, $p > .05$.

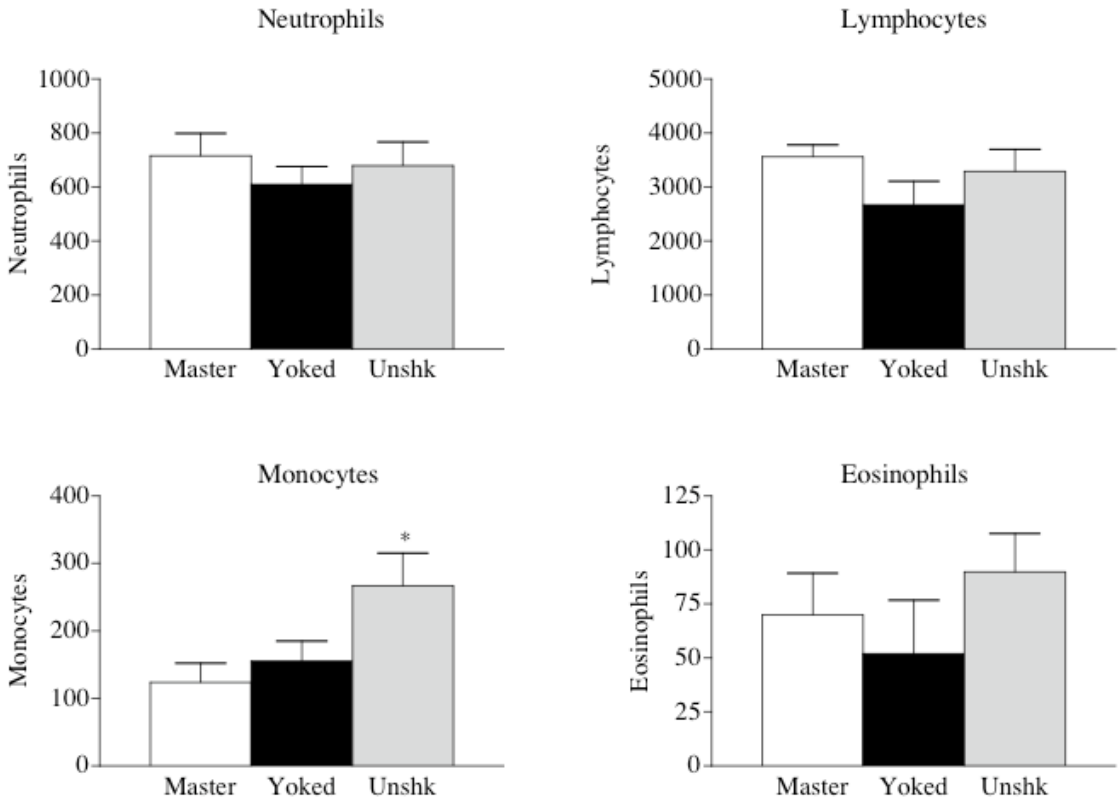

Figure 23. The effects of controllability on blood neutrophils, lymphocytes, monocytes and eosinophils 24 hours following the last training session.

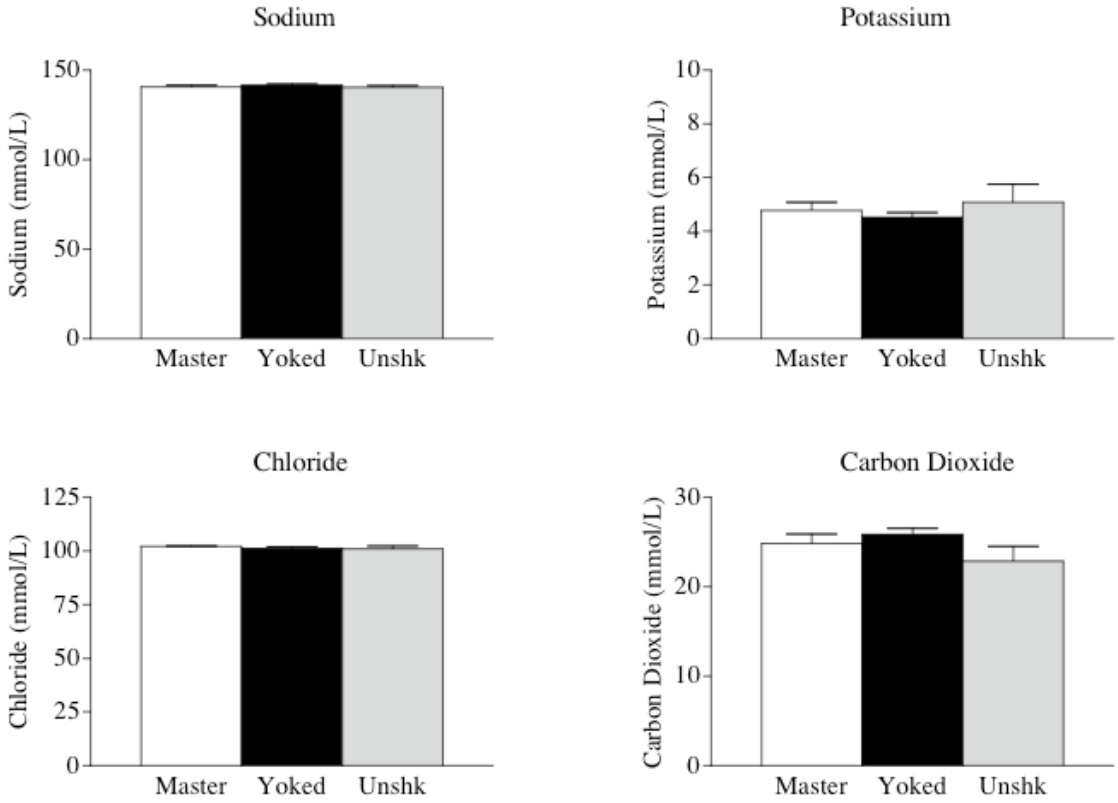

Figure 24. Results from the electrolyte panel for master, yoked, and unshocked subjects. Blood was analyzed 24 hours after the last instrumental training session.

Discussion

Experiment 4 produced some interesting and unexpected results. For example, controllable shock led to an increase in BBB locomotor scores but had no significant effect on any of the other biological outcomes assessed. Surprisingly, unshocked subjects exhibited higher corticosterone levels than both master and yoked subjects and this increase in corticosterone was accompanied by an increase in IL-6 in tissue from the injury site and an increase in the number of monocytes within the blood.

CHAPTER VII

SUMMARY AND GENERAL DISCUSSION

Impact of Contusion Injury

The amount and extent of secondary damage that occurs following spinal cord injury determines the amount of function retained. Many treatment strategies are therefore aimed at limiting the processes that contribute to secondary damage. Among these processes are vascular, biochemical, inflammatory, cellular, and molecular events. Factors that exacerbate these processes, like stress, may worsen functional outcome after injury. Thus, it is essential to understand how stress affects these processes. The current experiments begin to address this issue. Experiment 1 showed how contusion injury itself causes changes in several biological indices of stress including, corticosterone and norepinephrine levels, changes in spleen weight, and expression of the pro-inflammatory cytokines, IL-1β and IL-6. Three groups were used in this experiment to systematically address how contusion injury impacts these processes. They included anesthetized controls, subjects that only received a laminectomy (shams), and contused subjects. Overall, no differences were found among these groups for any of the indices assessed.

Very few studies have examined the impact of spinal cord injury itself on measures commonly used to assess the stress response, including changes in corticosterone and norepinephrine. To my knowledge, only one study by Popovich et al. (2001) has looked at the impact of contusion injury on corticosterone levels. In this study, they used a similar contusion model and had treatment groups comparable to the ones used here–anesthetized controls, laminectomy group, and contused group. They

found that corticosterone levels were significantly higher in subjects that received a contusion injury 1 day postinjury. However, by 3 days postinjury, corticosterone levels were not significantly higher than those from the laminectomy group. In Experiment 1, corticosterone levels were assessed 24 hours after injury in blood collected from the leg and again at 48 hours after injury when subjects were sacrificed. However, no differences were found at either timepoint. This discrepancy in findings may result from differences in sex and strain of rats used in the two studies. Female, Lewis rats were used in the Popovich study, whereas male, Sprague-Dawley rats were used in the current study. Several studies have demonstrated both sex and strain differences in corticosterone responses to stress, with females typically exhibiting a much more pronounced response (Chisari et al., 1995; Faraday et al., 2005).

Studies focusing on the impact of spinal cord injury on norepinephrine have generally assessed changes in norepinephrine at the injury site. Most of these studies have shown little or no significant changes in norepinephrine levels at the injury site (Naftchi et al., 1974; Rawe et al., 1977). Although norepinephrine levels were assessed in blood plasma in the current study, we also observed no significant changes.

Unlike corticosterone and norepinephrine, there have been a number of studies assessing the expression of pro-inflammatory cytokines after spinal cord injury. For the most part, these studies have shown significant increases in IL-1β and IL-6 at the injury site that emerge and peak within 6 to 12 hours (Hostettler & Carlson, 2002; Nesic et al., 2001; Nakamura et al., 2003; Wang et al., 1997, 2005). Typically, levels return to normal within 48 hours. Thus, it is not surprising that no effects were found in

Experiment 1, in which IL-1β and IL-6 levels at the injury site were not assessed until 48 hours after injury. Analyses at an earlier timepoint may reveal a different pattern of results.

Impact of Uncontrollable Shock

Uncontrollable shock was used as a stressor in Experiments 2-4 to determine how stress impacts several biological outcomes following spinal cord injury. Changes in spleen weight, corticosterone, and norepinephrine, which are known to change in response to stress, were measured to establish whether uncontrollable shock induces a stress response. We found that uncontrollable shock did produce a stress response characterized by a decrease in spleen weight and increases in corticosterone. These effects emerged within 24 hours and persisted for up to 7 days. These findings are in line with others, which have shown that stressors, particularly uncontrollable ones, produce similar effects (LeMay et al., 1990; Nguyen et al., 1998; O'Connor et al., 2003; Sumova & Jakoubek, 1989; Turnbull et al., 1994; Yamamotova et al., 2000; Zhou et al., 1993). In one study, footshocks, were used in a model of anticipation stress (Sumova & Jakoubek, 1989). Rats received footshocks over the course of 8 days. On the 9th day, they were placed in the chamber for 15 minutes but did not receive any shock. Spleen weight and corticosterone levels were assessed immediately thereafter. Subjects given footshocks had smaller spleens and exhibited higher corticosterone levels. Interestingly, these researchers showed that naloxone pre-treatment potentiated the decrease in spleen weight suggesting that the opioid system may preserve or protect the spleen from the consequences of stress. Naloxone, however, had no effect on changes in corticosterone.

Their finding that naloxone potentiated the reduction in spleen weight suggests that morphine pre-treatment may attenuate this effect. However, we found that morphine had no effect on the decrease in spleen weight observed after uncontrollable shock. In fact, morphine itself caused a reduction in spleen weight in unshocked subjects (Figure 13B). Because morphine is a μ-opioid receptor agonist, our findings imply that an opioid receptor system other than the μ opioid receptor system is involved in the preservation and/or protection of the spleen during stress.

Although uncontrollable shock impacted both spleen weights and corticosterone levels, it had no effect on plasma norepinephrine. This is surprising given that this neurotransmitter has been heavily implicated in the behavioral deficit in learned helplessness. Weiss and colleagues (1976) developed a neurochemical model that centered on norepinephrine as the primary candidate to account for the behavioral effects observed after uncontrollable shock. They maintained that exposure to uncontrollable shock led to a dysfunctional noradrenergic system, resulting partially from depletion of norepinephrine in limbic brain areas. Motor behavior in animals subjected to uncontrollable shock would be reduced as a result of this depletion, causing the animals to respond less to their environment. It is important to note that it is not the shock *per se* that disrupts the noradrenergic system, but rather the uncontrollable aspect of the shock that produces the deficit. Subjects given control over the shock do not exhibit any changes in noradrenergic function (Weiss et al., 1980).

Here we showed that plasma norepinephrine was not elevated at any of the timepoints assessed. These results may have emerged because we failed to assess

norepinephrine at an early enough timepoint following shock treatment. For example, others have shown that uncontrollable shock causes an increase in plasma norepinephrine, but this effect is transient, lasting less than 30 minutes after shock termination (Swenson & Vogel, 1983; Weyers, Bower, & Vogel, 1989). The earliest timepoint examined in the current experiment was 6 hours after shock exposure. Examination at an earlier timepoint may show increases in plasma norepinephrine. Furthermore, this transient increase in plasma norepinephrine is often associated with a subsequent decrease in brain norepinephrine. Uncontrollable shock typically causes a decrease in norepinephrine in regions such as the locus coeruleus, hypothalamus, and hippocampus that is accompanied by an increase in its metabolite, 3-meth-oxy-4-hydroxy-phenylethylene glycol sulfate (MHPG-SO_4; Anisman et al., 1987; Heinsbroek et al., 1991; Lehnert et al., 1984; Swenson & Vogel, 1983; Weiss et al., 1980). We did not examine norepinephrine content in the brain, thus future studies are warranted to determine whether our shock procedure produces similar effects.

Impact of Morphine

Experiment 3 used morphine to determine the effect of eliminating the psychological experience of pain caused by uncontrollable shock. Previous work from our laboratory showed that morphine did not prevent the consequences of uncontrollable shock, but rather exacerbated its effect on some measures of recovery, including sensory function and lesion size (Hook et al., 2007). Here, we show that morphine again did not attenuate the effects of uncontrollable shock in contused subjects. Morphine treatment failed to prevent the impairment of locomotor recovery, the decrease in spleen weight,

and the increase in corticosterone that results from shock exposure. In some cases morphine potentiated these responses and even caused similar changes in the absence of uncontrollable shock. For example, like uncontrollable shock, it caused a significant increase in corticosterone, IL-1β and IL-6. These results are consistent with those from other studies, which have shown that comparable doses of morphine increase plasma corticosterone within 1 hour (Budziszewska et al., 1999; Laorden & Milanes, 1999; Mellon & Bayer, 2001; Milanes et al., 1993; Simon et al., 1975). Our work suggests that plasma corticosterone levels stay elevated for up to 24 hours after morphine treatment.

Morphine also elevates circulating levels of both IL-1β and IL-6 (Bertolucci, Perego, & Simoni, 1996; Johnston et al., 2004; Houghtling & Bayer, 2002; Houghtling et al., 2000). Upregulations of IL-1β and IL-6 mRNAs, as well as protein levels, have also been demonstrated in the brain and spinal cord (Johnston et al., 2004; Zubelewicz et al., 1999). The mechanism underlying this effect remains largely unknown; however, it requires an intact adrenal cortex because morphine does not increase IL-6 in adrenalectomized animals (Houghtling & Bayer, 2002; Houghtling et al., 2000). Corticosterone may play a role because increases in IL-6 are often accompanied by increases in corticosterone (Houghtling & Bayer, 2002), as was the case in the current study. However, this correlation does not address the directionality of the effect. The corticosterone response may drive the IL-6 response or vice versa. Evidence exists for both hypotheses. For example, IL-6 is known to activate the HPA axis, causing the release of corticosterone (Lenczowski et al., 1999). On the other hand, increases in corticosterone are believed to precede the elevation in IL-6 (Houghtling & Bayer, 2002).

Still other evidence suggests that the increase in corticosterone is independent of IL-6 elevation (Houghtling & Bayer, 2002; Ray, LaForge, & Sehgal, 1990).

The morphine-induced increase of IL-1β and IL-6 at the injury site suggests that morphine treatment, like shock, causes an elevation of activated microglia and macrophages in the spinal cord. This finding contradicts other studies, which have shown that, in general, morphine suppresses immune function. For example, morphine has been shown to inhibit chemotaxis in monocytes (Perez-Castrillon et al., 1992; Stefano et al., 1993), microglia (Chao et al., 1997) and granulocytes (Makman, Bilfinger, & Stefano, 1995). Morphine treatment also decreases natural killer (NK) cell activity (Hoffman et al., 1995; Mellon & Bayer, 2001; Shavit et al., 1986; Weber & Pert, 1989), decreases blood lymphocyte proliferation (Fecho et al., 1996; Hoffman et al., 1995; Houghtling et al., 2000; Mellon & Bayer, 1998, 2001), reduces polymorphonuclear cell activity, and decreases the number of phagocytic cells, as well as, suppresses phagocytic activity (Tubaro et al., 1987). Acute morphine treatment also affects more sophisticated immune responses such as antibody production (Jankovic & Radulovic, 1992; Veljic et al., 1992). The exact mechanism through which these effects are mediated remains unknown, although both the autonomic nervous system and the HPA axis have been implicated (Fecho et al., 1996; Flores, Dretchen, & Bayer, 1996; Mellon & Bayer, 2001). Further studies will be needed to determine if morphine enhances or suppresses immune function in our spinal cord injury model. Regardless, the finding that morphine increases corticosterone and pro-inflammatory cytokines expression at the injury site has

important clinical implications because it suggests that a routinely used clinical treatment could contribute to secondary damage after spinal cord injury.

Impact of Controllability

The last experiment determined if controllability could prevent the consequences of shock. Experiment 4 failed to show any significant differences between master and yoked subjects on any of the biological variables tested. Unshocked subjects, however, exhibited higher corticosterone than both master and yoked subjects and this increase was accompanied by an increase in IL-6 at the injury site and monocytes in the blood. These results are puzzling given our previous findings in unshocked controls. However, it should be noted that the procedure used in this experiment differed from that used in the previous experiments. For example, tailshock was used previously to administer uncontrollable shock, whereas legshock was used in the current experiment. Another difference concerns the amount of time the unshocked subjects were restrained in the tube. Subjects in Experiments 2 & 3 only experienced 6 minutes of restraint, whereas subjects in Experiment 4 were restrained for 30 minutes for two consecutive days. There were also differences in how these subjects were restrained. Subjects in Experiment 4 were retrained in tubes that allowed their hindlimbs to hang freely and accordingly have to be secured using a wire belt in. The tubes used in the other experiments provided a flat surface on which the subject could lay and have barriers at the end to prevent escape; a wire belt was not required. These differences in procedure may help to explain why unshocked subjects had high corticosterone levels. They do not, however, explain why unshocked subjects, that received the exact same treatment as the master and yoked

subjects with the exception of extensive shock exposure, differed significantly from these two groups. Future studies will be needed to resolve these issues. One solution is to include a home cage control to assess the impact of the restraint that is included in the paradigm.

Mechanism

Figure 25 outlines a model that illustrates how exposure to uncontrollable shock may lead to impaired recovery of function. In this model, which is a modified version of that proposed by Griffis et al. (2006), nociceptive input caused by uncontrollable tailshock activates nociceptors, which relay the pain signal to the spinal cord through A-delta and C peripheral nerve fibers (Cousins & Power, 1999; Soledad Cepeda & Carr, 1996). The signal is then projected to the brainstem reticular formation through ascending pain pathways like the spinothalamic tract and on to the hypothalamus and locus coeruleus to activate the stress axes (Soledad Cepeda et al., 1996; Yaksh, 1999). Projections to the limbic structures induce the negative emotions, like fear and anxiety, which are associated with pain. The limbic and forebrain areas initiate descending inhibitory signals, which originate in the periaqueductal gray (PAG) project to the rostral ventro-medial medulla (RVM) and to the dorsal horn of the spinal cord. These descending signals cause the release of endogenous opioids, serotonin, and norepinephrine that decrease the nociceptive input (Pasero, Paice, & McCaffery, 1999). Activation of the HPA and sympatho-adreno-medullary stress axes also causes the release of other endogenous opioids that modulate the pain signal at both the brain and spinal cord level (Fields & Basbaum, 1999; Soledad et al., 1996).

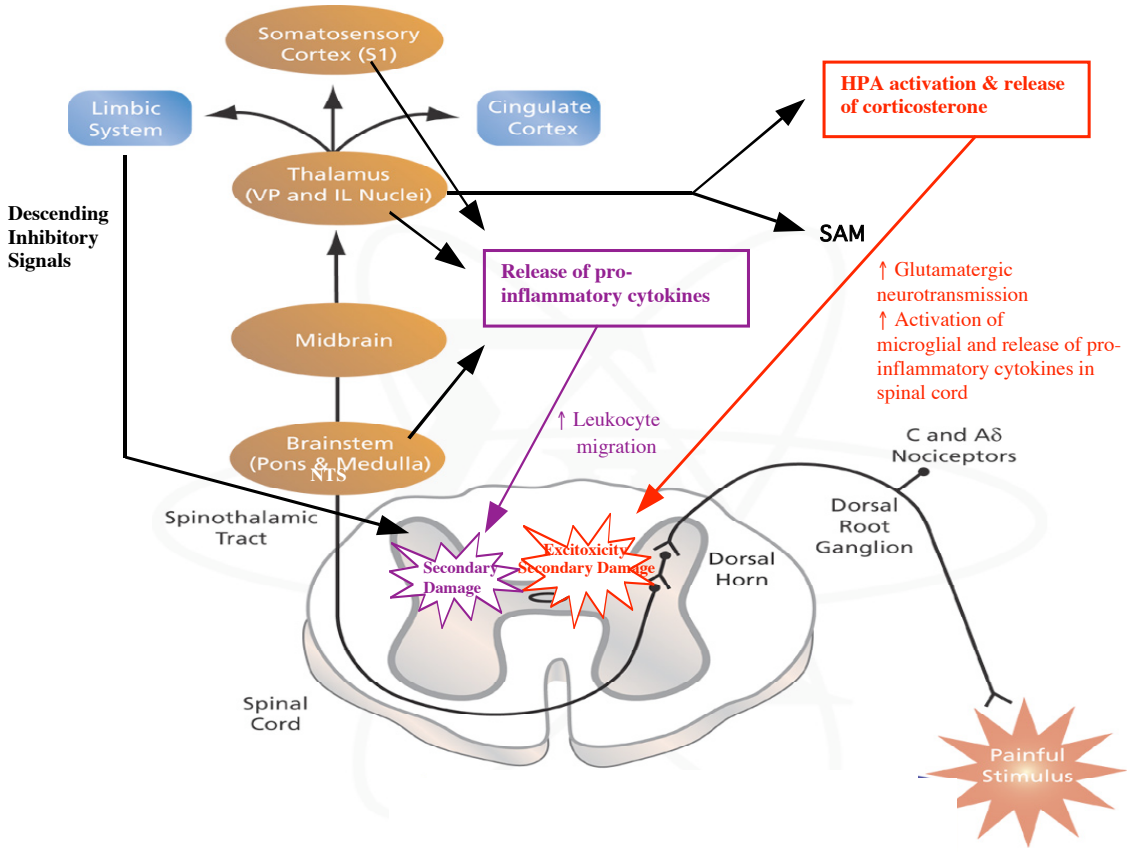

Figure 25. Outlines two mechanisms through which uncontrollable shock may contribute to cell loss and loss of recovery of function. Uncontrollable shock activated nociceptors, which in turn activate A-delta and C fibers. These peripheral nerve fibers transmit the signal to the spinal cord where it is relayed to the brain via the spinothalamic tract. The signal travels through the brain stem to the midbrain up to thalamus and cortex. Activation of limbic structures cause descending inhibitory signals to be sent to the dorsal horns of the spinal cord. Activation of the hypothalamus causes the release of corticosterone into the bloodstream. Corticosterone travels to the spinal cord where it enhances glutamatergic neurotransmission and excitotoxicity that results in cell loss in the spinal cord (red arrow). Activation of the nucleus tractus solitarius (NTS), hypothalamus, and cortical areas results in the release of pro-inflammatory cytokines, which stimulate macrophages and enhance leukocyte migration to the spinal cord, resulting in potentiation of secondary damage (purple arrow). Adapted version of a figure that appears on Sigma-Aldrich.com.

The model contains two separate pathways through which shock may produce its effects. The first involves activation of the HPA axis and subsequent release of corticosterone (red box and arrows). It is well recognized that many types of stressors cause an increase in glucocorticoids, like corticosterone, that can have both direct and indirect, modulatory effects on other systems. These effects can be beneficial in some cases but detrimental in others. For example, glucocorticoids have been shown to both enhance and impair learning and memory processes (For a review see Sapolsky, 2003). Moderate, short-lived increases are correlated with beneficial effects of glucocorticoids, whereas high, prolonged increases are associated with negative effects. Uncontrollable shock caused a prolonged increase in corticosterone in the current study that was associated with an impairment of locomotor function. This loss of motor function may have resulted as a direct consequence of corticosterone potentiating excitoxicity within the spinal cord. The physical and psychological component of uncontrollable shock would cause activation, and possibly dysregulation, of the HPA axis, which in turn results in the release of corticosterone. Corticosterone has been shown to enhance glutamatergic neurotransmission in the hippocampus by increasing extracellular excitatory amino acid concentrations (Lowy, Gault, & Yamamoto, 1993; Virgin et al., 1991) and potentiating NMDA receptor function (Bartanusz et al., 1995; Krugers et al., 1993; Mulholland et al., 2006; Takahashi et al., 2002; Weiland, Orchinik, & Tanapat, 1997). In the presence of injury, these effects contribute to excitoxicity and exacerbate cell loss that can attenuate recovery of function. A similar effect may be observed in the spinal cord, which also contains glucocorticoid receptors (Cintra, Molander, & Fuxe,

1993; Clark, Maclusky, & Naftolin, 1981; Takasaki et al., 2005). Furthermore, corticosterone-induced activation of the NMDA receptor may also lead to microglia proliferation and the release of pro-inflammatory cytokines within the spinal cord. Nair and Bonneau (2006) showed that four days of restraint stress induced proliferation of microglia in the brain. They concluded that this effect was a result of corticosterone-induced activation of the NMDA receptor because the effect was reversed by blocking corticosterone synthesis, glucocorticoid receptors, and NMDA receptors. Their work suggests that stress-induced release of corticosterone can activate microglia, resulting in a pro-inflammatory response within the CNS that could potentiate inflammatory conditions. Potentiation of the inflammatory response after spinal cord injury could contribute to secondary damage and greater loss of function.

The other mechanism through which uncontrollable shock may disrupt recovery of function is illustrated in purple in Figure 25. In addition to activating the stress axes, uncontrollable shock is also believed to activate pathways associated with "sickness behavior", resulting in increases in IL-1β and a potentiated immune response. Maier and Watkins (1998) suggest that the nociceptive signal enters into the central immune activation, or sickness behavior, pathway in the brainstem. The signal travels through the brainstem to the nucleus tractus solitarius (NTS), hypothalamus, and cortical areas to induce the release of pro-inflammatory cytokines by macrophages and other immune cells. Pro-inflammatory cytokines then activate receptors on leukocytes and endothelial cells to increase cell adhesion molecule (CAM) expression, and thereby enhance leukocyte migration to areas of inflammation (Dinarello, 2003; Wang, Czura, & Tracey,

2003). The process by which leukocytes and lymphocytes are trafficked to areas of tissue damage and inflammation has been well characterized and involves four steps (Butcher, 1991; Springer, 1994). The first of which is called "rolling" and involves formation of weak bonds between endothelial CAMs, which are expressed in response to trauma, infection, and hormones, and CAMs on leukocytes. In this process, leukocytes roll end over end through the blood, repeatedly breaking the CAM bonds. Step 2, referred to as "activation," occurs when leukocytes are exposed to chemokines such as IL-8 and macrophage inflammatory protein that bind receptors on their cell surface, causing a phenotype switch into an activated leukocyte. Integrins on the cell surface undergo a confirmational change that fosters stronger bonds. "Tethering" occurs in Step 3 when these integrins form strong bonds with endothelial CAMs, stabilizing their proximity to the endothelial surface. At the same time, inflammatory mediators that increase vascular permeability are released. Extravasation then occurs as the leukocyte moves from the blood vessel to the tissue. In the process, they become activated, differentiated effector cells capable of producing chemokines, pro-inflammatory cytokines and other mediators that exacerbate the inflammatory process by attracting more leukocytes and increasing blood flow and vascular permeability (Goldsby, Kindt, & Osborne, 2000; Rohde & Lee, 2003). Enhancement of leukocyte migration may be detrimental to recovery of function given that neutrophils and macrophages are thought to contribute to the secondary damage that occurs following spinal cord injury.

Increases in CAM expression and subsequent leukocyte migration may be one mechanism through which stress exacerbates a number of inflammatory diseases in both

humans and animal models (Griffis et al., 2006). These diseases include arthritis (Chover-Gonzalez et al., 2000; Seres et al., 2002), asthma (Liu et al., 2002; Matalka, 2003; Okuyama et al, 2007), psoriasis (Buske-Kirschbaum et al., 2007; Folks & Kinney, 1992; Weigl, 2000), multiple sclerosis (Sieve et al., 2004), inflammatory bowel disease (Tadakazu et al., 2007), and artherosclerosis (Blankenberg, Barbauex, & Tiret, 2003). Viswanathan & Dhabhar (2005) showed that acutely stressed mice had a higher number of neutrophils, macrophages, natural killer cells, and T cells that infiltrated an area of inflammation in comparison to nonstressed animals. They also showed that these same animals had higher levels of lymphotactin, which is a lymphocyte specific chemokine and TNF-α, both of which increase leukocyte trafficking. Similar effects have been noted after stress in individuals with psoriasis (Buske-Kirschbaum, 2007) and rheumatoid arthritis (Liote et al., 1996). Others have shown that increased CAM expression is associated with disease exacerbation in rheumatoid arthritis (Liote et al., 1996), inflammatory bowel disease (Nielson, Brynskov, & Vainer, 1996), as well as, coronary artery disease (Blankenberg et al., 2003; Huo & Ley, 2001; Lutters et al., 2004; Rohde & Lee, 2003). Collectively, these findings suggest that increased CAM expression and enhanced leukocyte migration may contribute to inflammatory diseases.

The results from Experiment 2 support both of the proposed mechanisms discussed above. For example, uncontrollable shock caused an increase in corticosterone within 24 hours that persisted throughout all the timepoints tested and this increase was accompanied by a decrease in locomotor recovery. This finding supports the idea that uncontrollable shock activates the HPA axis to induce the release of

corticosterone and this increase may be detrimental to recovery. Further studies will be needed to determine whether the increase in corticosterone observed after uncontrollable shock facilitates glutamatergic neurotransmission, leading to excitoxicity within the spinal cord. Work in the hippocampus would serve as a good model for these studies. Several techniques have been used in that work to address the issue directly. These include the use of microdialysis to measure the amount of extracellular excitatory amino acids after exposure to stress (Lowy et al., 1993), in vitro analysis of cytotoxicity in response to glucocorticoid exposure (Mulholland et al., 2006), and measuring reuptake of excitatory amino acids by astrocytes following exposure to glucocorticoids in vitro (Chou, 1998). The issue could also be addressed indirectly by determining the impact of blocking corticosterone and/or NMDA receptors on cell death and recovery of function in subjects given uncontrollable shock. Our laboratory has already begun to examine the effects of a NMDA receptor antagonist.

Experiment 2 also showed an up-regulation of IL-1β and IL-6 at the injury site following uncontrollable shock. Given that microglia and macrophages are the major source of these cytokines (Hartlage-Rubsamen, Lemke, & Schliebs, 1999; Garabedian, Lemaigre-Dubreuil, & Mariani, 2000), this suggests an increase in activated macrophages in the spinal cord. While this finding implies that there is an increase in immune cell migration to the site of injury, it does not conclusively address how uncontrollable shock produces this effect and whether it contributes to impaired recovery of function. Again, further studies will be needed. These studies will need to examine whether the uncontrollable shock regimen used in the current experiments increases pro-

inflammatory cytokines, particularly IL-1β, in the brain like other stressors (Deak et al., 2005; Nguyen et al., 1998; O'Connor et al., 2003). Studies will also need to show that uncontrollable shock increases proteins involved in leukocyte trafficking, including CAMs, and will need to definitively show an up-regulation of immune cells at the injury site.

Summary and Conclusion

In summary, contusion injury *per se* had no impact on spleen weight, corticosterone, norepinephrine, or pro-inflammatory cytokines 2 days postinjury. Contused subjects given uncontrollable shock exhibited a locomotor deficit, had smaller spleens, higher levels of corticosterone, and increases in IL-1β and IL-6 at the injury site. These effects emerged within 24 hours of shock treatment and persisted for up to 7 days. Morphine treatment did not attenuate the effects of uncontrollable shock but instead potentiated them. Controllability also appeared to have no impact on the consequences of uncontrollable shock. However, due to the type and duration of the restraint used in the master/yoke paradigm, additional studies will be needed to further investigate this issue. Two mechanisms were proposed to explain the impact of uncontrollable shock on recovery of function. One involved corticosterone-induced excitotoxity and the other involved cytokine enhancement of leukocyte migration to the injury site. Additional studies will be needed to determine which pathway(s) is/are involved in the effects of uncontrollable shock on recovery of function after spinal cord injury.

Our work has important implications for recovery of function after spinal cord injury. Using a clinically relevant model of injury, we have shown that uncontrollable nociceptive input induces a stress response that may result in greater loss of function. In human spinal cord injury, this nociceptive input may take the form of broken limbs lacerations resulting from the accident or even neuropathic pain that results from the injury itself. Regardless of the source, it is imperative to prevent this type of input to the spinal cord. According to our findings, morphine treatment may not be the best treatment strategy. In fact, morphine may actually contribute to the loss of function after spinal cord injury. Further studies will be needed to identify clinical interventions that can prevent the nociceptive input and preserve recovery of function.

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